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| 14. ABSTRACT Legumain is a recently discovered and only known asparaginyl endopeptidase that is well conserved throughout the biologic kingdoms. We have demonstrated that legumain is highly and inappropriately expressed in 100% human breast cancer specimens as well as murine breast cancer models. We demonstrated that an inactive prototype doxorubicin derived prodrug incorporating a succinyl blocked substrate peptide removable by legumain was effectively activated and tumoricidal in human breast cancer models. We designated this prodrug legubicin. Legubicin is not cytotoxic until activated by legumain due to reduced ability to enter cells and blocked binding to DNA. These properties led to increased tumor exposure and much reduced drug accumulation in normal tissues when administered in vivo. It has markedly reduced cardiac and myelosuppressive toxicities compare to doxorubicin. In this grant application we propose to further develop this prodrug strategy as a potential treatment for breast cancer. | | | | | |
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Legubicin a Tumor-activated Prodrug for Breast Cancer Therapy

Background

Using positional gene expression profiling and high density tissue arrays, we have discovered that legumain is highly expressed by a majority of solid tumors, including 100% breast carcinomas examined [1]. Immunohistochemical analysis of tumor tissues reveals that legumain expressed by tumor cells as well as endothelial cells and tumor associated macrophages (TAM), cells constitute tumor microenvironment. In tumor microenvironment, legumain is present on cell surface in the tumor microenvironment and functional due to the acidic condition existed there. Legumain is a recently identified protease, a distinct member of the C13 family of cysteine proteases [2]. It is well conserved throughout the biologic kingdoms, found first in plants, subsequently in parasites, as well as mammals as an endopeptidase. Legumain is active in acidic pH condition and quickly inactivated under neutral pH. It has a very restricted specificity requiring an asparagine at the P1 site of substrates. Its novel specificity supports that it may be implicated in limited proteolysis consistent with limited proteolytic activation of protease zymogens, as well as selected proteins and peptides. The human legumain gene encodes a preproprotein of 433 amino acids. Mouse legumain shares 83% homology with the human protein [3]. Cells expressing legumain have enhanced migratory and invasive properties. A correlation between tumor invasion and metastasis with some cysteine endopeptidases (particularly cathepsins B and L) has been observed [4]. Legumain is critical in the activation of cathepsin B, D, and H [2, 5, 6]. We and others showed legumain activates the zymogen progelatinase A, an important mediator of extracellular matrix degradation [1] [7]. The reported inhibitory effect of cystatins on tumor cells [8, 9] is consistent with the involvement of legumain and perhaps other cysteine proteases in tumor invasion and metastasis. Tumor invasion and metastasis are the major determinants of lethality, linked to 90% of human cancer deaths [10]. The high level of legumain expression by breast cancer cells and associated cells in the tumor microenvironment coupled with its unique specificity makes it an attractive candidate for prodrug therapy for breast cancer. We reported a doxorubicin derived prodrug prepared by incorporating a peptide extension of the amino group of doxorubicin resulting in an inactive compound unless hydrolyzed to leucine-doxorubicin by an asparaginyl endoprotease [1]. This compound, legubicin, resulted in complete tumor growth arrest and eradication in a model of human breast carcinoma without toxicity, such as weight loss, myelosuppression, and cardiac toxicity in contrast to doxorubicin treated mice. Doxorubicin and related compounds are the mainstay of breast cancer chemotherapy, however its application is limited by its toxicity. In contrast to doxorubicin, legubicin do not enter cells efficiently until activated by cell surface legumain in the tumor microenvironment. Pharmacokinetics analysis and tissue distribution study support tumor specific localization and activation of legubicin. As consequence, tumor accumulation and exposure to legubicin is greatly enhanced and organ exposure to chemotherapeutic agent is reduced. In organs containing cells that normally express legumain, such as kidney and liver, no injury was evident. Legumain expressed by kidney and liver cells are present in lysosomes and not

secreted. Extracellular legumain will be quickly inactivated by neutral environment in plasma and normal tissues. Legubicin demonstrated improved efficacy profile and therapeutic index vs doxorubicin by targeting neoplastic cells as well as endothelial cells and TAM in the tumor microenvironment therefore represents a promising candidate as a first line molecularly targeted chemotherapeutic agent replacing doxorubicin for the breast cancer treatment.

Body

The funding from W81XWH-05-1-0318 provided critical support for us to pursue research in translational medicine and experimental therapeutics in the area of breast cancer. We have made significant progress in this grant cycle.

1. Development of legumain activated prodrug, legubicin, as an anti-breast cancer treatment.

A. In vivo characterization of legubicins for preclinical efficacy, therapeutic protocol advancement and safety in a panel of rodent and human breast tumors in vivo. (Months 1-36)

An advanced candidate of the prodrug was tested in variety of breast cancer models and demonstrated significant effect against primary tumor growth and metastasis. The preclinical efficacy and safety was reported in a recent Cancer Research paper titled "Targeting Cell-impermeable Prodrug Activation to Tumor Microenvironment Eradicates Multiple Drug Resistant Neoplasms" will appear in Jan. 15th issue of Cancer Research. W81XWH-05-1-0318 provided critical support for the research and was gratefully acknowledged (PDF file of the paper is attached as appendix).

Most significantly, the prodrug demonstrated significant efficacy preventing metastasis in both spontaneous and experimental metastasis.

Selective ablation of tumor associated macrophages as a mean to suppress metastasis and angiogenesis is integrated in this section.

Primary and metastatic neoplastic development both depend on the tumor microenvironment. Tumor growth requires angiogenesis and the tumor associated macrophages (TAMs) are key producers of growth factors, such as VEGF, that induce angiogenesis and support tumor cell survival. Legumain is an asparaginyl endopeptidase which is specifically over-expressed on the surface of endothelial cells and TAM in tumor stroma in addition to the neoplastic cells. Using a doxorubicin-based prodrug specifically activated in the tumor stroma by legumain, we showed that effective killing of both TAMs and endothelial cells. Massive tumor cell death followed the death of TAMs and endothelial cells. The subsequent collapse of tumor micro-vasculatures resulted in complete tumor growth inhibition without any apparent toxicity. Targeting resident cells in the tumor microenvironment has distinctive advantages since both TAMs and endothelial cells are non-transformed and much more sensitive to

chemotherapeutic agents versus tumor cells which are frequently multi-drug resistant. The prodrug treatment effectively reduced TAMs in tumors and resulted in significant reduction in angiogenic factors and other growth factors that support tumor cell survival. The anti-angiogenic effect is demonstrated by reduced vessel density in treated tumors. Consequently, the prodrug therapy exerts combined antiangiogenic and anti-tumor effect. The targeted prodrug activation permits metronomic dosing at an effective level critical to prevent the recovery of TAMs and endothelial cells; it effectively seizes the therapeutic windows created by normalization of tumor vasculature following angiogenic factor depletion. Consistent with the importance of TAMs in angiogenesis tumor progression, the accumulation of TAMs proceeds apparent angiogenic responses at the site of metastasis. More importantly, administration of this prodrug significantly reduces metastasis in metastatic models. The prodrug suppresses spontaneous metastasis and significantly reduces circulating tumor cells as demonstrated by blocking metastasis following surgical removal of primary tumors, a clinically relevant setting, and extends survival of the host without toxicity. Our findings indicate TAMs play a critical role in tumor development and metastasis. The potent *in vivo* efficacy suggests that metronomic dosing of legumain-activated prodrug represents a novel anti-cancer strategy targeting multiple steps during tumor metastasis and angiogenesis.

Targeting TAMs or molecules in the tumor microenvironment which attract them and mediate their function represents a novel anti-tumor strategy. TAMs consist of a polarized M2 (CD206+, F4/80+) macrophage population with little cytotoxicity for tumor cells because of their poor production of nitric oxide and proinflammatory cytokines. TAMs also possess poor antigen presenting capacity and effectively suppress T cell activation. In fact, TAMs actually promote tumor cell proliferation and metastases by producing a wide range of growth and pro-angiogenesis factors, metalloproteinases and also partake in circuits that regulate the function of fibroblasts in the tumor stroma.

The selection of Legumain as a target for tumor therapy is based on the fact that the gene encoding this enzyme is highly up-regulated in many murine and human tumor tissues (Liu, Sun et al. 2003), but absent or only present at very low levels in all normal tissues from which breast tumors arise. Legumain is a distinct member of the C13 family of cysteine proteases (Chen, Dando et al. 1997). It is well conserved throughout the biologic kingdoms, found first in plants, subsequently in parasites, as well as mammals as the only known asparaginyl endopeptidase (AEP). It has a very restricted specificity requiring an asparagine at the P1 site of substrates. In this regard, we recently discovered that Legumain is heavily expressed by TAMs in tumor tissues by using gene expression profiling and immunohistochemistry. Importantly for our studies, TAMs are express high levels of legumain in the tumor microenvironment. In contrast, classical macrophages of M1 phenotype, that perform key immune-surveillance functions, do not express Legumain. Consequently, targeting TAMs that over express Legumain does not interfere with the biological functions of normal macrophages, including cytotoxicity and antigen presentation (Sica, Sacconi et al. 2002).

We have designed a Cell-impermeable Tumor Microenvironment Activated Prodrug (TMEAP) that specifically activated only by legumain on the cell surface and target TAMs. We demonstrated that selective killing of Legumain-expressing TAMs down regulate a wide variety of tumor growth factors, pro-angiogenesis factors and enzymes released by these macrophages and lead to inhibition of tumor angiogenesis as well as growth and metastasis. Our data support critical role of TAM in promote tumor cell entering blood stream. This approach may represent a novel cancer therapy against a wide spectrum of solid tumors, since TAM infiltration is evident in high percentage of cancers.

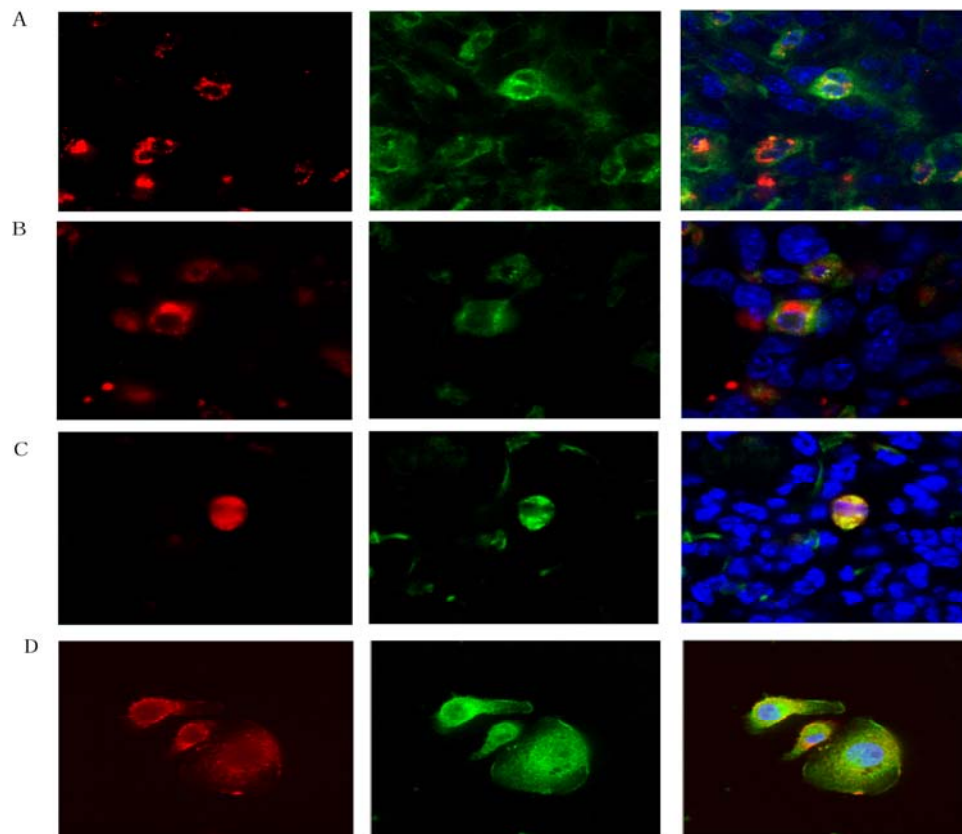


Figure 1. Legumain is highly expressed in Tumor Associated Macrophage (TAM) and present on cell surface of M2 macrophages.

A. Legumain (Green) is expressed in both tumor cells and TAM in the 4T1 tumor central area. Macrophages are identified by CD68 (Red) positivity. **B.** Legumain (Green) primarily expressed in TAM in the 4T1 tumor periphery area. Legumain is Green and CD68 is Red. **C.** Tumor Associated Macrophage (red) in early 4T1 lung metastasis express high level of legumain (Green). **D.** M2 macrophage induced by treating RAW cells with IL-4, IL-10, and IL-13 (10 ng/ml). Legumain is stained Green and F-Actin is Red.

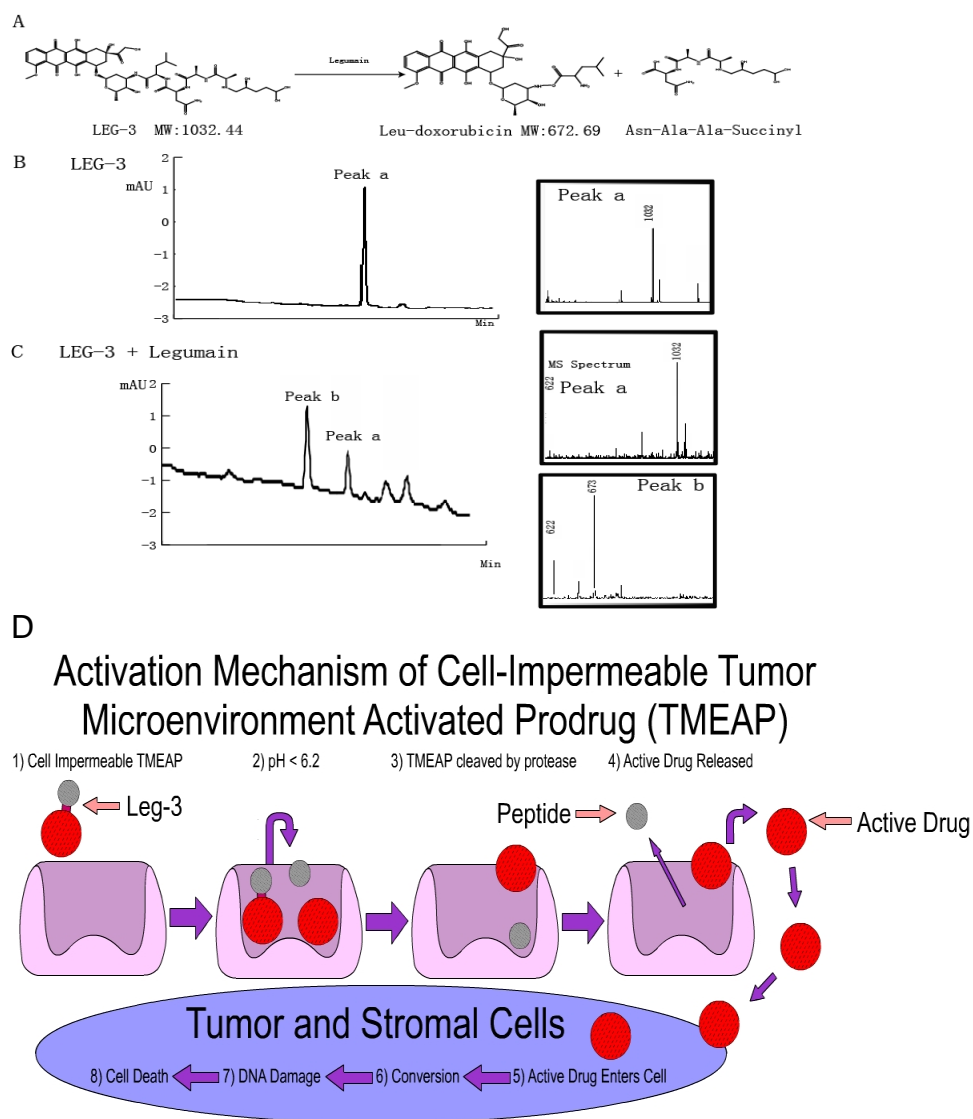


Figure 2. LEG-3 is a cell-impermeable tumor microenvironment activated prodrug (TMEAP) specifically activated by legumain.

A. LEG-3 structure and expected product by legumain activation. **B.** LC-Mass analysis of LEG-3 compound. **C.** LC-Mass analysis indicate LEG-3 cleavage by legumain produce active Leu-doxolubicin (Peak b). **D.** Mechanism of cell-impermeable targeting tumor microenvironment targeting TAMs.

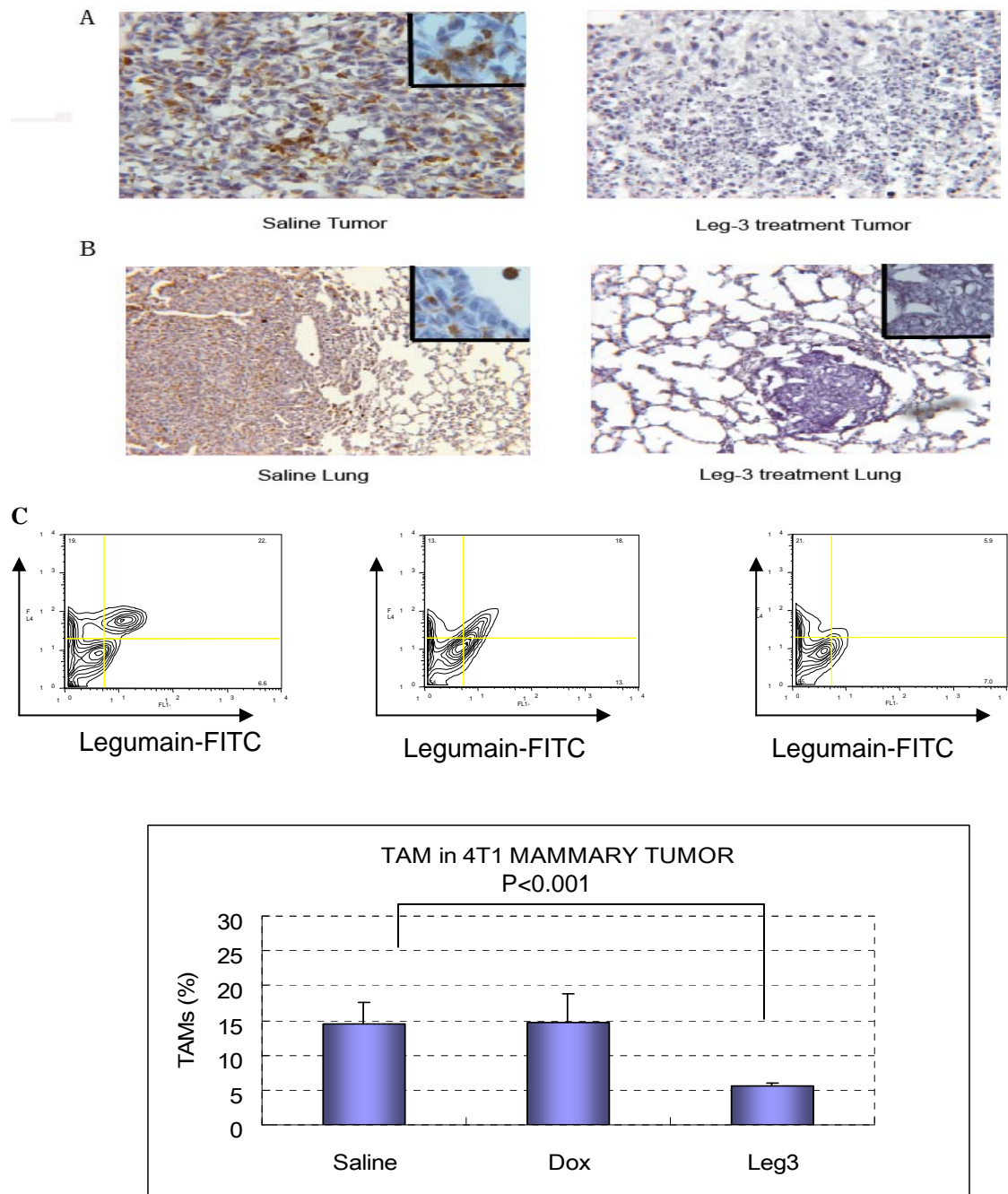


Figure 3. LEG-3 treatment decreases TAM population in the tumor microenvironment.

A. TAM infiltrates, identified by anti-CD68 staining (brown) in primary 4T1 tumor tissue is eliminated by LEG-3 treatment. **B.** LEG-3 eliminated TAMs in 4T1 lung metastases. **C.** FACS analysis of TAMs in the control and LEG-3 treated tumor stroma. *** $p < 0.001$ (between LEG-3 and Saline). In contrast, LEG-3 treatment does not affect F4/80+ and CD206+ myeloid derived cells in spleen.

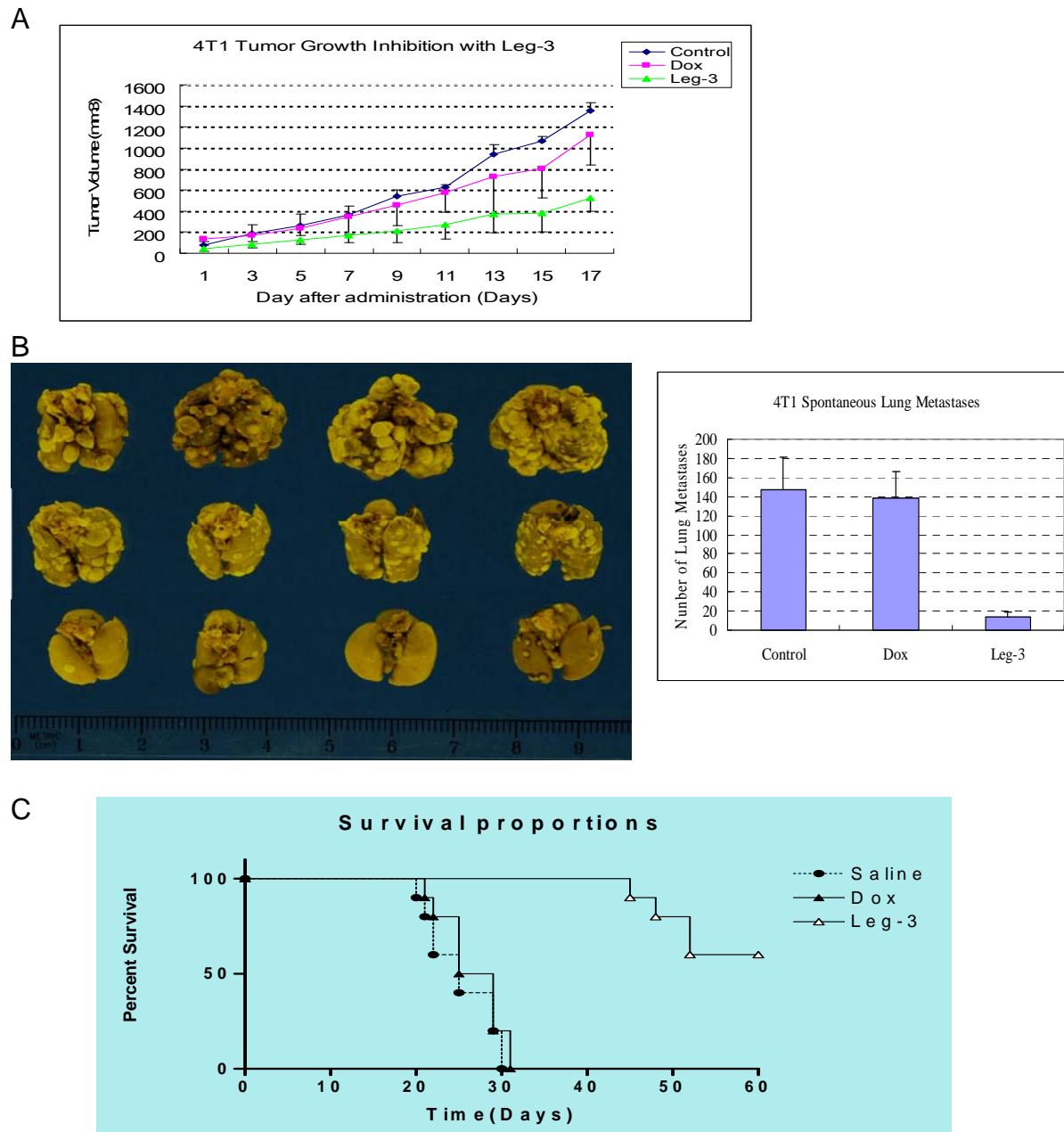


Figure 4. Efficacy of LEG-3 in 4T1 murine breast carcinoma model compare to doxorubicin.

A. *In vivo* effect of LEG-3 (49.4 $\mu\text{mol/kg}$) compare to Doxorubicin (3.4 $\mu\text{mol/kg}$) and mock treatment control. Tumor volume are expressed as mean \pm SD. $**p < 0.002$ (LEG-3 and Dox); $***p < 0.001$ between LEG-3 and saline. **B.** Effect of LEG-3 on 4T1 spontaneous lung metastasis. LEG-3 treatment significantly reduces metastasis $***P < 0.01$. **C.** Survival analysis of LEG-3 treated mice. $***p < 0.001$ is calculated using the Cox-Mantel log-rank test.

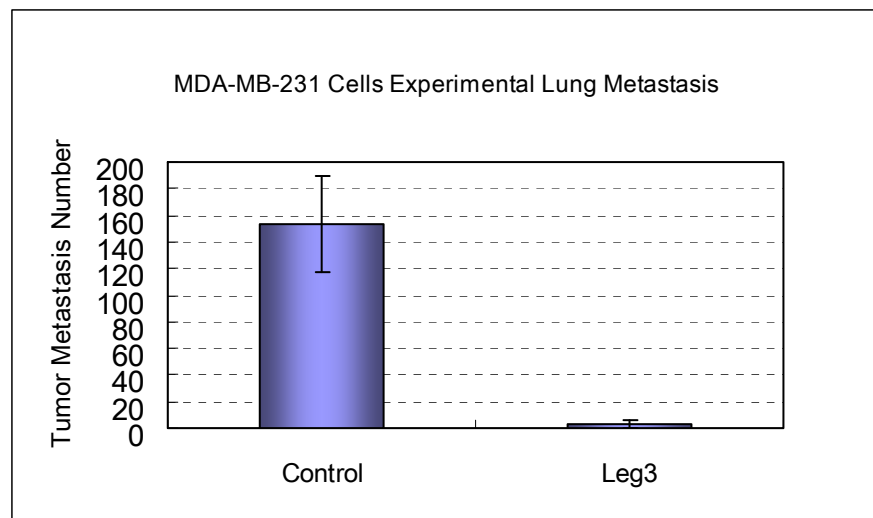
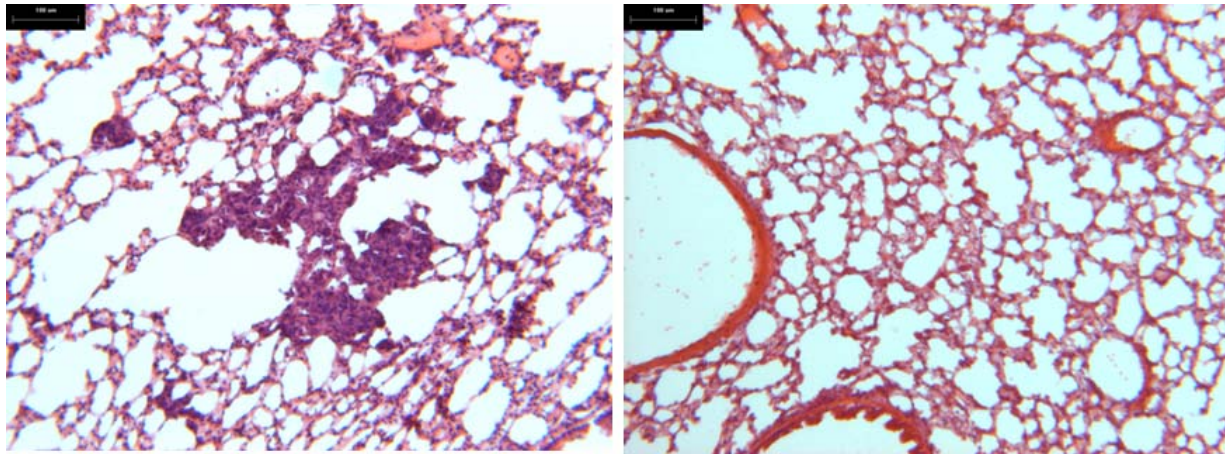


Figure 5. Efficacy of LEG-3 in experimental lung metastasis models.

MDA-MB-231 human breast cancer model generated in SCID mice. Representative lung colonies of the tumor masses were counted in every 50 μm series of 10 5 μm sections. Sections of lungs from control groups (left), leg-3 treatment (right) are shown. *** $P < 0.001$.

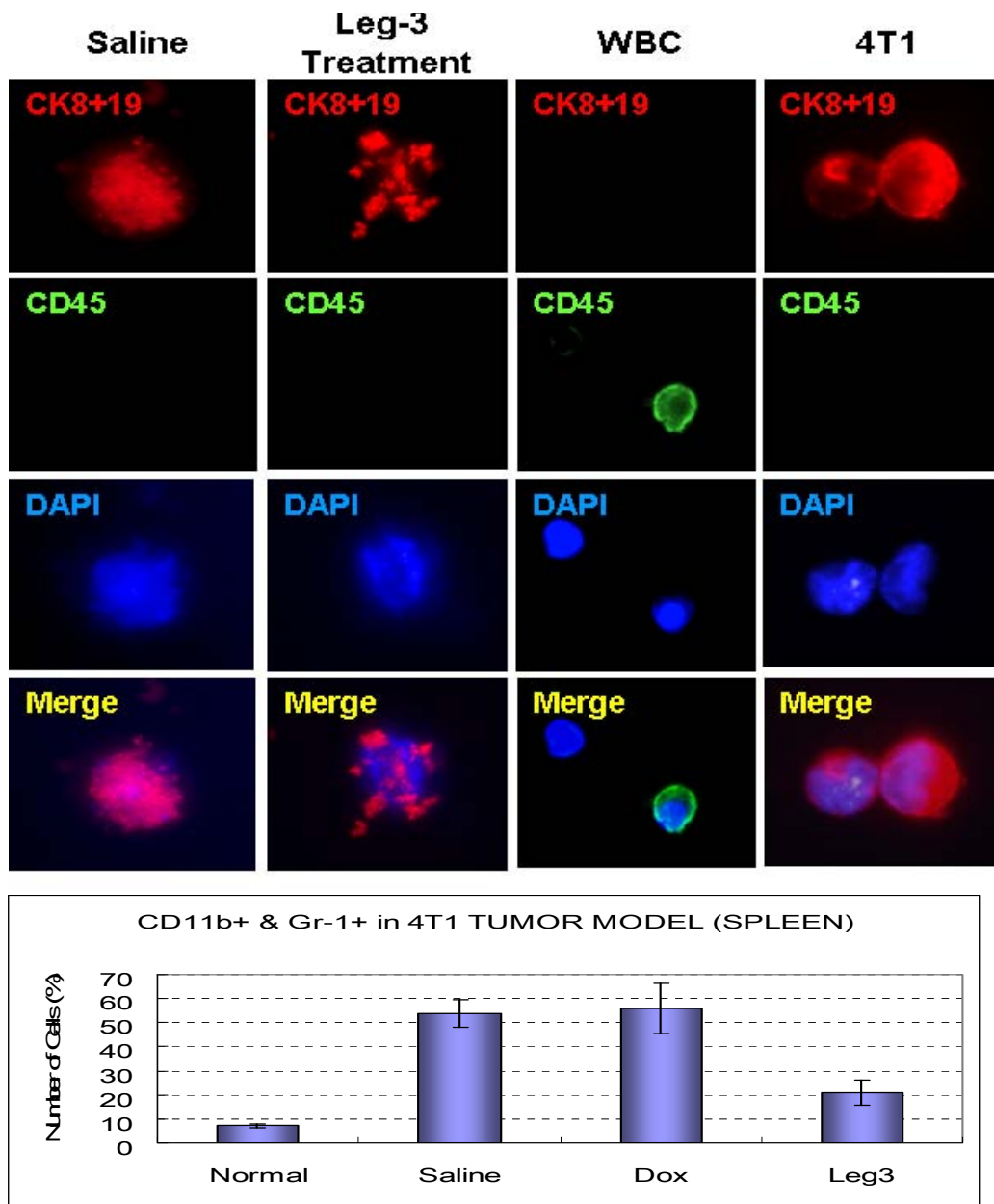


Figure 6. Elimination of TAM significantly reduces circulating tumor cells (CTCs) in 4T1 breast cancer model.

A. CTC identified in the blood of LEG-3 treated and control tumor bearing mice. CTC is CK 8+19 (red) positive and CD 45 (green) negative. Nuclear is blue. **B.** The number of CTC in control and leg-3 groups (n=6). *** $P < 0.001$.

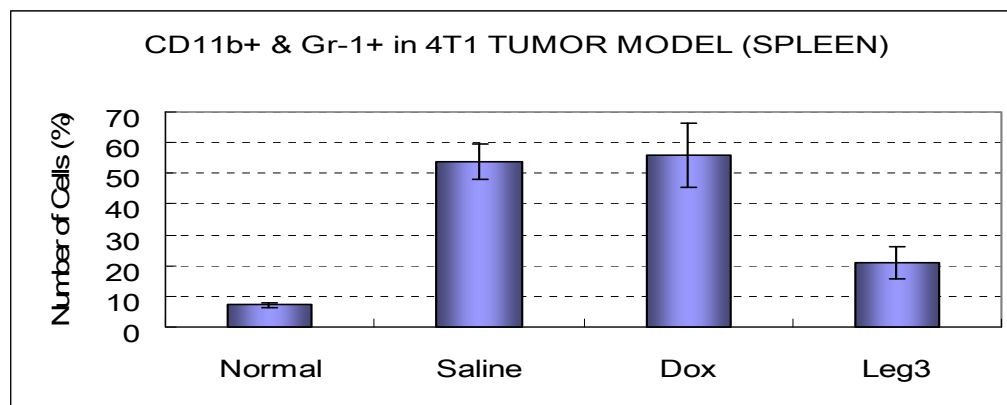
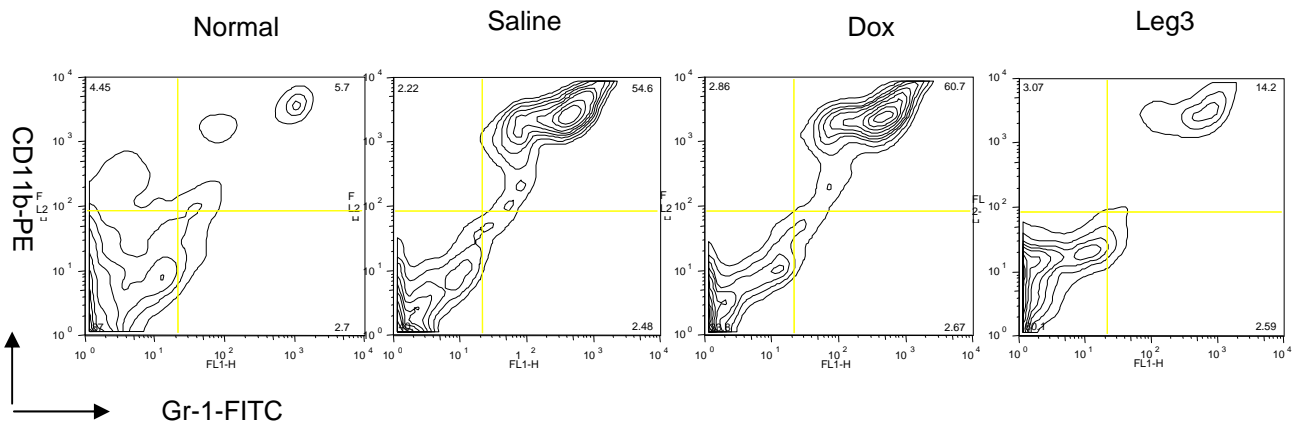


Figure7. LEG-3 treatment decreases CD11b+Gr-1+ cells in the tumor microenvironment.

Upper: CD11b+Gr-1+ cells was identified by anti-CD11b staining (brown) in primary 4T1 tumor tissue is eliminated by LEG-3 treatment. **Lower:** FACS analysis of CD11b+Gr-1+ cells in the control and LEG-3 treated tumor stroma. *** $p < 0.001$ (between LEG-3 and Saline and Dox).

In conclusions of this section, legumain is highly expressed on TAM surface. A doxorubicin-based prodrug specifically activated by legumain selectively kills TAMs. The prodrug treatment resulted in significant reduction in angiogenic factors, prevents metastasis without toxicity. Most importantly, it significantly extended survival of the host. Selective ablation of TAMs significantly reduces circulating tumor cells reduces Gr-1+/CD11b+ Myeloid-derived suppressor cells (MDSC). These data support that TAMs play critical roles in tumor development and metastasis and represent an effective target for therapy

Materials and Methods:

Cell Culture and Animal model:

The murine breast cancer 4T1 cells were cultured in DMED medium with 2 mM L-glutamine containing 10% FCS and 1mM NEAA in CO₂-free environment. MDA-MB-231 human breast cancer cells (ATCC, Rockville, MD) were cultured in DMED medium with 2 mM L-glutamine containing 10% FCS and antibiotics in CO₂-free environment.

To estimate the metastatic ability of the hybrids, we used a lung colonization model. Briefly, parental and hybrid cells were prepared as single-cell suspensions in sterile PBS at a concentration of 5×10^6 cells/ml, and a volume of 100 μ l was injected i.v. via the tail vein into 6-week-old female (BALB/c). Animals were sacrificed on a day between days 30 and 35, when the majority of the control mice became moribund. The lungs were fixed in Bouin's solution for picture and counting of lung tumor metastasis. by microscope.

All mice were purchased from The Scripps Research Institute Rodent Breeding Facility. Tumor induction was performed by s.c. injection of 5×10^5 4T1 cells in the right flank of six-week-old BALB/c mice. Three different groups of four animals were treated between days 9 and 27 after tumor induction. Treatment involved 100- μ l i.p. injections of either Saline alone (control group) and Leg-3 treatment group was given 1mg/ 100 μ l of leg-3 in Saline by i.p. injection every days for two weeks. Tumor volumes of treated animals were measured every two-day starting on day 9 by microcaliper measurements (volume = length \times width \times width/2). As soon as the tumor volume reached 1400 mm³ in the control groups (on day 30), euthanasia was performed and lungs were removed and fixed in the Bouin's solution. The yellow surface metastatic tumors were counted were counted by anatomy microscope.

Statistical significance between treatment groups was determined by two-tailed Student's *t* tests using Microsoft EXCEL software.

All of the animal experiments were approved by the Institutional Animal Care and Use Committee of the Scripps research Institute before the experiments were started.

Statistics: The statistical significance of differential findings between experimental groups and controls was determined by Student's *t* test. Findings were regarded as significant, if 2-tailed *P* values were less than 0.05. Evaluate the survival of mice was used by Kaplan-Meier analysis

Enrichment of CTCs:

Briefly, 0.5 ml of blood was collected from mouse into a acid citrate dextrose (ACD) tube (Beckton Dickinson, Franklin Lakes, NJ), and washed once with AS1 solution (AVIVA Biosciences Corp, San Diego, CA), followed by lysis of RBC using AS2 solution (AVIVA Biosciences Corp, San Diego, CA). The sample was washed once again with AS1, and subsequently subjected to AVIVA micromagnetic particles conjugated to anti-mouse CD45 (eBiosciences, San Diego, CA) to remove white blood cells (WBC) by means of a magnetic separation stand (Progenia, Madison, WI). Supernatants were transferred into a new eppendorf tube and spun down using a microcentrifuge. Resulting cells were spotted onto a slide.

Immunofluorescence staining protocol is similar to that previously described (Lin et al., 2007). Briefly, cells were fixed by 2% paraformaldehyde (PFA) in Tris buffered saline (TBS, pH 7.2) for 40 min at room temperature, followed by permeabilization using 0.1% Triton X-100 for 10 min. Sample was blocked with 2% BSA + 2% rabbit serum in TBS for 1 hr at room temperature, and subsequently subjected to sequential antibody incubation with anti-mouse cytokeratin 8 + 19; biotinylated rabbit anti-rat IgG; Texas Red labeled streptavidin; and FITC conjugated anti-mouse CD45. Above antibodies were diluted with blocking solution, and washing was performed using 0.2% BSA in TBS after each antibody incubation.

Immunocytochemical staining:

Frozen specimens were cut at 8 μ m in a cryostat, fixed 4% Paraformaldehyde for 10 min and incubated PBS containing 0.3% H₂O₂ and 0.3% immunized normal sera. Antibodies were diluted with phosphate buffered saline with 1% bovine serum albumin. The monoclonal antibody rat anti mouse CD31 (clone MEC133; BD Pharmingen) and rat anti F4/80 (AbD Serotec) were diluted 1:100 for overnight at 4°C and bound antibody was detected with biotinylated rabbit anti rat IgG and streptavidin-peroxidase complex using DAB as the substrate.

Flow cytometry:

Tumor-Associated Macrophages (TAMs) were isolated from successfully leg3 treated mice and control mice 4T1 breast cancer tissue. Staining was performed with PE-labeled anti-F4/80 Ab (eBioscience) in combination with 1st Ab, sheep anti-mouse legumain and 2nd Ab, rabbit anti sheep -conjugated FITC. TAMs bearing high levels of CD206⁺ and F4/80⁺ were quantified by 2-color flow analysis. PE-labeled CD11c and Gr-1⁺ conjugated FITC antibodies (eBioscience) was used with 1:50 dilution for one hour at room temperature.

immunohistochemistry

4T1 mice lung tissue were embedded in the OCT. Sections were cut for 8 μ m by

Cryostep. Slides were brought to room temperature and dried by hear dryer for 20 min. Fixed in cold Acetone at -20°C for 10min. After incubation with normal goat serum blocking solution for 30 minutes, incubate sections with Avidin D solution for 15 minutes. Rinse briefly with PBS, then incubate for 15 minutes with biotin solution (Avidin/Biotin blocking kit, Vector). Subsequently, the slides were incubated with a cocktail of first primary antibodies for overnight (biotin-conjugated rat anti-mouse CD68 at 1:100 dilution and sheep anti mouse legumain at 1:100 dilution from stock solution). The second antibodies were incubated with Rhodamine Avidin DCS (Red) and Fluorescein anti-sheep (green) (Vector Laboratories) for 40 minutes. Nuclear staining was performed DEPI (10ug/ml) for 10 minutes, then mount cover slip with anti-fade medium

A PCT patent titled "Inhibiting Tumor Cell Invasion, Metastasis, and Angiogenesis" was filed Nov. 29, 2006. (PDF file is attached).

B. Legumain specificity explored through phage displayed substrate peptide library and synthetic peptide. (Months 1-6)

Due to the success with the current candidate compound, the construction of phage library is delayed. However we are constructing a peptide array with the new instrumentation available at protein core facility and this is applicable to additional proteases with interest in breast cancer biology and is not restricted with legumain alone.

C. Improving chemistry and synthesis of legubicins, and efficient analysis of designed peptidyl and pseudopeptidyl derivative structures for improved efficacy and safety of legubicin analogues using in vitro assays. (Months 1-36)

We have synthesized different versions of the prodrug including dimmer the prodrug. Legumain (and other proteases) activated prodrugs with paclitaxel are synthesized (see patent applications). Some of these candidate compounds have been be assessed for their in vitro and in vivo efficacies in breast cancer models how ever most of these works area outside the scope of current grant.

2. Characterization of the molecular cell biology of legumain in breast cancer biology.

A. Producing legumain knockdown and over-expression cells and investigate the effect of legumain knockdown and over-expression on breast cancer cells migration invasion in vitro. (Months 1-12)

We have produced multiple breast cancer cell lines that are over-expressing legumain as well as expressing legumain shRNA that suppresses legumain expression. Our data support the role of legumain in cell migration and invasion. Therefore this aim is accomplished. These work led to new hypothesis of mechanisms that legumain modulate tumor microenvironment.

B. Investigate the effect of legumain knockdown and over-expression on breast cancer invasion/metastasis in animal models. (Months 6-18)

Continuing from the last aim, We discovered that legumain: $\alpha_v\beta_3$ protease complex regulate cell surface proteolysis.

We demonstrated that legumain is present in the tumor microenvironment binding to cell surface integrins, such as $\alpha_v\beta_3$ and $\alpha_5\beta_1$. The legumain: $\alpha_v\beta_3$ complexes are predominantly at the front of migrating cells. Binding of legumain to $\alpha_v\beta_3$ integrins significantly enhances its activity towards physiologic substrates, such as pro-MMP2 and pro-cathepsin L. Therefore, integrins are both receptor and cofactor of legumain. Inhibition of legumain activity by a high affinity cell-impermeable asparaginyl endopeptidase inhibitor (AEPI) suppresses angiogenesis and tumor cell invasion *in vitro* and *in vivo*. Systemic administration of AEPI reduces tumor growth, vascular density, and tumor invasiveness in breast cancer models. Importantly, this treatment inhibits both spontaneous and experimental lung metastasis. We will (a) examine effect of modulating legumain activity on tumor cell behavior *in vitro* and *in vivo*; (b) evaluate AEPI efficacy and safety in a panel of rodent and human cancer models; (c) examine synergistic effect of cell-impermeable AEPI and chemotherapeutic agents.

The unique asparaginyl endopeptidases, legumain, is induced by hypoxia and transported to the front of invading cells. Legumain forms complex with integrins exclusively on the cell surface of lamellipodia and invadopodia. Binding of legumain to its cofactor integrins significantly enhances its ability to activate pro-MMP2 and pro-cathepsin L. Therefore the legumain:integrin complex physically and functionally define an invasive cell front surface (ICFS) where it regulates a network of matrix-processing enzymes. Inhibition of legumain:integrin affects matrix remodeling and suppresses angiogenesis and cell invasion. Importantly, systemic administration of a potent, cell impermeable asparaginyl endopeptidase inhibitor (AEPI-1) inhibits tumor angiogenesis and invasive growth. The treated tumors demonstrated profound disorganization of collagens and elastic fiber. This treatment prevented both spontaneous and experimental lung metastasis suggesting the activity of legumain:integrin is required for metastasis. Our findings indicate legumain:integrin is an important regulator of cell invasion and matrix remodeling and an effective therapeutic target for cancer.

Neoplasms in humans arise via a multi-stage process that is reflected by genetic and epigenetic alterations that drive progressive transformation. In addition to limitless replicative capacity and evasion of anti-growth signals, the malignant cancer cells acquire ability to sustain angiogenesis and invasion of surrounding tissues (1). This transformation is at least partly driven by hypoxia and lack of nutrients in the local tumor microenvironment. It is well established that hypoxia induces tumor invasive growth and angiogenesis in tumors (2, 3). These two processes are also intimately linked at mechanistic levels by both involving activation of extracellular proteases and changes in cell adhesion to the surrounding environment. Proteases are up-regulated and the

protease inhibitors are down-regulated. Inactive zymogens are converted to active enzymes. These matrix degrading proteases are associated with invasive cell surfaces directly or via binding to cell surface molecules. Changes in integrin expression are also evident. During angiogenesis and tumor invasion, integrin expression profile shifted from those that favor ECM present in normal epithelium and endothelium to that preferentially binding to the transitory and degraded stromal components produced by proteases, such as $\alpha v\beta 3$ and $\alpha 5\beta 1$.

Legumain is a distinct member of the C13 family of cysteine proteases (4). It is well conserved throughout the biologic kingdoms, found first in plants, subsequently in parasites, as well as mammals as an endopeptidase. It has a very restricted specificity requiring an asparagine at the P1 site of substrates. Previously we have demonstrated legumain is up-regulated in high percentage of solid tumors and play an important role in tumor invasion and metastasis. Legumain can activate both matrix metalloproteinase and cysteine proteases. Recently, there are evidences suggesting cathepsin cysteine proteases are critical effectors of invasive tumor growth and angiogenesis during tumorigenesis. These findings put the focus on the roles of cysteine proteases in human cancer.

To define the role of legumain in the molecular proteolytic pathways involved in cancer development, we examined legumain expression under hypoxia that is common in the tumor microenvironment. We sought to profile the emergence of legumain expression and enzymatic activity during tumor growth in both primary tumors as well as in metastasized sites. We presented evidence that integrins, particularly $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins, are cell surface receptor and cofactor of active legumain enzymes. Both legumain and $\alpha v\beta 3$ integrin expressions are up-regulated by hypoxia. The coordinated emergence of legumain and $\alpha v\beta 3$ integrins not only localizes legumain to invasive cell surface, importantly, the $\alpha v\beta 3$ integrins also provided co-factor functions that regulate legumain enzymatic activity and pH dependency. These data implicated that formation of the legumain: $\alpha v\beta 3$ proteolytic complexes on the invasive surface of tumor and angiogenic endothelial cells represents a critical early step in tumorigenesis. Consistent with these observations, inhibition of legumain activity using asparaginyl endopeptidase inhibitor (AEPI) led to reduced endothelial tube formation *in vitro* and angiogenesis in a matrigel plug assay *in vivo*. Treatment with AEPI also blocked angiogenesis, tumor invasive growth and metastasis *in vivo*. These results indicate the coordinated induction of legumain and $\alpha v\beta 3$ integrins by hypoxia and the cell surface presentation of legumain: $\alpha v\beta 3$ at the leading edge of migrating cells is important for tumor invasive growth and angiogenesis. Here we characterized a new cell surface proteolytic complex that is functionally linked to hypoxia in the tumor microenvironment. The legumain: $\alpha v\beta 3$ is capable of activating multiple families of proteases that are proven important for tumor progression. Collectively these data suggest that cell surface legumain: integrin complex in the tumor microenvironment represent a promising target for cancer therapy. We demonstrated the efficacy of a novel strategy for cancer therapy using AEPI that targets multiple critical steps during tumor progression including tumor invasive growth, metastasis, and angiogenesis.

Legumain expression is induced by hypoxia.

Hypoxia is an important factor in the tumor microenvironment. We examined legumain expression in human tumor cells and endothelial cells under both normoxic and hypoxic culture conditions. Legumain expression was detected with western blot analysis. In both MDA-MB231 human breast carcinoma cells, Panc-1 pancreatic carcinoma cells and Human umbilical vein endothelial cells(Huvec-C), legumain expression is induced in cells cultured under hypoxic conditions (1% O₂), and its level continues to rise as the duration hypoxic exposure increases (Figure 1a). HUVEC express very little legumain under normal culture conditions, but legumain expression is significantly enhanced when the cells are cultured under hypoxic conditions for 72 hours (Figure 1a). Hypoxia induced legumain expression is accompanied by the appearance of cell surface legumain (Figure 1b).

Integrins are cell surface receptor for legumain.

Legumain contains a RGD domain that is frequently present in integrin binding proteins. To determine whether legumain binds to integrins, we performed immunoprecipitation with a panel of anti-integrin antibodies in MDA-MB231 cells that express low level of legumain in culture. Legumain is co-precipitated with anti- α v, α v β 3, β 3, and β 1 antibodies, suggesting α v β 3 is one of the candidate integrins (Figure 1c). To further characterize the legumain: α v β 3 interaction, co-immunoprecipitation was performed with anti-legumain antibody and detected with anti- α v integrin antibody (Figure 2d). In addition, legumain was detected when anti- α v β 3 was used as the immunoprecipitating antibody (Figure 2d). The presence of legumain: α v β 3 complex is supported by extensive co-localization of legumain and α v β 3 in human breast and pancreatic cancer cells grown in nude mice (Figure 1e and f).

The α v β 3 integrins is a co-factor of cell surface legumain activity.

We determined the effect of binding of integrins on legumain enzymatic activity. Legumain activity was assessed using a fluorescent substrate of legumain. With increasing concentration of α v β 3 integrin protein, there is a dramatic increase of legumain amidolytic activity suggesting α v β 3 integrins is not only a cell surface receptor of legumain. It is also a co-factor of legumain activity (Figure 2a). Binding of legumain with α v β 3 can increase legumain activity nearly 100 fold (Figure 2b). Further, binding of legumain to α v β 3 also affect its pH dependency. The activity of legumain: α v β 3 complex are measured in different pH conditions and compared to that of legumain. Binding of legumain to α v β 3 shifts its peak activity from pH 5.2 to pH 6.0. The legumain: α v β 3 is active near pH 7, under which legumain will be inactivated (Figure 2b). These properties will significantly increases legumain activity at mild acidic conditions that is existed in the extracellular space in the tumor microenvironment. The enhanced legumain amidolytic activity and shifted pH dependency indicates the cell surface legumain: α v β 3 complex is likely the primary target enzyme of tumor microenvironment activated prodrug.

We assessed the activity of legumain: $\alpha\text{v}\beta 3$ against legumain physiologic substrate MMP2 (Figure 2c) and Cathepsin L (Figure 2d). These results indicate that the legumain: $\alpha\text{v}\beta 3$ complex is an important modulator of pericellular proteolysis during tumor invasive growth and angiogenesis.

Asparaginyl endopeptidases inhibitors are highly specific and well tolerated.

Four asparaginyl endopeptidases inhibitors (AEPI) were characterized using recombinant legumain in an amidolytic activity assay with fluorescent substrate (Supplemental a). Both AEPI-1 and AEPI-2 have IC_{50} at 20 nM and 21 nM respectively, the IC_{50} of AEPI-3 is 34 nM, while IC_{50} of AEPI-4 is 158 nM. We evaluated the cytotoxicity of AEPI-1 (Figure 2e) was assessed in both wild type 293 cells (CC_{50} , 320 μM) and 293 cells expressing legumain (CC_{50} , 430 μM). No apparent cytotoxicity was observed until the 100 μM AEPI-1 was used (Supplemental b). AEPI-1 are highly specific to Legumain and don't influence the activity of cathepsin B, D, L, MMP2 and caspase 3, 8 activity (Figure 2f).

Legumain: integrin complex forms exclusively at the surface of lamellipodia and invadopodia.

Next, we performed Immunohistochemical staining of MDA-MB-231 human mammary carcinoma cells with both anti- $\alpha\text{v}\beta 3$ and anti-legumain antibodies. Very little legumain and $\alpha\text{v}\beta 3$ integrins are expressed in cell cultured under normal conditions (Supplemental c). However, the level of legumain and $\alpha\text{v}\beta 3$ integrins are both dramatically elevated under hypoxia and transported to cell surfaces where these proteins co-localize extensively. In migrating cells, the legumain: $\beta 1$ (Figure 3a), legumain: $\alpha\text{v}\beta 3$ (Figure 3b) and legumain: $\alpha 5\beta 1$ complexes are predominantly present at the leading edge of the cells. The Legumain presenting at the leading edge of the cells are also found in the PC-3 and Huvec-c cells (Figure 3c).

Legumain regulates a network of powerful matrix-processing enzymes, we expect the presence of Legumain: Integrin on cell surface will be very discreet and highly regulated. We found Legumain: Integrin complex are exclusively formed at the front surface of lamellipodia and invadopodia. Legumain co-localizes with multiple integrin subtypes such as $\beta 1$, $\alpha\text{v}\beta 3$ and $\alpha 5\beta 1$. Legumain co-localizes with integrins extensively in human breast, pancreatic carcinomas grown in nude mice indicating a important role of legumain: integrin in invasive tumor growth. To study whether AEPI-1 blocks the Legumain at the front surface of lamellipodia and invadopodia in vitro, we synthesize the specific AEPI-1-FITC compound (Figure 3d). The AEPI-1-FITC also presents at the leading edge of the cancer cells indicating AEPI-1 can block Legumain function on cell surface by forming AEPI-1: Legumain: Integrin complex (Figure 3e).

Legumain: Integrin regulates matrix remodeling at cell invasive front surface.

We assessed the ability of legumain: integrin to modulate matrix remodeling at the invasive cell front surface using FITC labeled DQ collagen. In Hypoxia condition, legumain enhance processing of collagen IV at the invasive cell front surface and it is

inhibited by AEPI-1, consistent with potent legumain:integrin activity to activate MMP2 and cathepsin L.

Legumain is critical for angiogenesis.

The effect of AEPI-1 on endothelial cell function was assessed *in vitro* in Matrigel endothelial cell tube formation assay. AEPI-1 suppressed HUVEC tube formation in a dose dependent manner (Figure 4a). Inhibition of tube formation was apparent at 500 nM and was completely inhibited at 1000 nM. In contrast, addition of recombinant legumain dramatically promoted and accelerated the tube formation (Figure 5b). HUVEC tube formation was observed as early as 5 hours after assay initiation versus under normal conditions 24 hours are required for the vascular tubes to form.

Next, the effect of AEPI-1 on angiogenesis was assessed in a mouse aortic sprouting assay. We tested the AEPI-1 in two settings. First, AEPI-1 was added at the start of the experiment to determine its effect on initiation of vessel sprouts. AEPI-1 inhibited FGF-2 induced vessel sprouts in a dose dependent manner (Figure 4c). In the second assay, AEPI-1 was added at the third day when sprouts are already formed. In the setting we try to determine whether asparaginyl endopeptidase activity is also required for the continued extension of vessel growth. Addition of AEPI-1 reduced extension of established vascular outgrowth suggesting the continued involvement of legumain in vessel development. These observations are consistent with the ability of legumain to activate proteases and its association with integrins such as $\alpha v\beta 3$ that are critical for angiogenesis.

We further investigated the effect of AEPI-1 on angiogenesis *in vivo* using a Matrigel plug assay. Matrigel containing FGF-2 was injected subcutaneously into Balb C nu/nu mice. AEPI-1 significantly inhibited FGF-2 induced vessel formation in the plug (Supplemental e). Next, we examined AEPI-1 effect on angiogenesis induced by cancer cells by including human breast cancer cells (MDA-MB-231) in the Matrigel. The cancer cells produced a spectrum of angiogenic factors and induced robust angiogenic vessel growth that is inhibited by AEPI-1 (Figure 4d) as indicated by gross appearance, hemoglobin content (Figure 4e) and histology (Figure 4f), indicating inhibition of asparaginyl endopeptidases activity affect angiogenesis induced by a wide range of angiogenic factors.

AEPI-1 disrupts tumor matrix remodeling and tumor invasive growth.

Next, we investigated the effect of AEPI-1 on tumor growth and invasion in a human mammary carcinoma model established in Balb C nu/nu mice. The MDA-MB-231 cells grew aggressively *in vivo* and form a tumor that is similar to human infiltrating ductal carcinoma with apparent glandular differentiation. The tumor invaded locally into surrounding tissues and skeletal muscles. The AEPI-1 not only suppressed tumor growth, it also blocked the tumor differentiation into a more invasive form (Figure 5d).

Next, we investigated whether pharmacologically inhibit legumain: integrin proteolytic complex will affect tumor growth. Affect of AEPI-1 treatment on tumor growth was assessed in 4T1 primary tumor models that are generated by s.c. injection of 5×10^5 4T1 cells in the right flank of six-week-old BALB/c mice. Daily administration of AEPI-1 suppresses tumor growth (Figure 5a). Vascular density is reduced in treated groups and the tumor lacks apparent invasion into surrounding tissues (Figure 5d).

Legumain activity is essential for distant metastasis.

Significantly, the AEPI-1 treatment blocked spontaneous metastasis of 4T1 tumor to lungs (figure 6h). The effect of AEPI-1 on metastasis is also evaluated in an experimental metastasis models by injecting 4T1 cells (1×10^5) suspended in 0.1 ml serum free medium into the tail vein of six-week-old female BALB/c mice. Treatment of AEPI-1 (100ug/day) given i.v. over two weeks also significantly reduced experimental metastasis in 4T1 murine mammary carcinoma (Figure 6i). Collectively, these data support the role of legumain: $\alpha v \beta 3$ complex in tumor development and indicate asparaginyl endopeptidase inhibitors are effective in suppressing angiogenesis, tumor invasive growth and metastasis therefore a promising candidate for cancer therapy.

The development of capabilities of cancers to invade and metastasize is the cause of 90% of all human cancer death (sporn 1996), and remain a great challenge for cancer research and treatment. Invasion and metastasis employ complex processes and genetic and biochemical mechanisms is incompletely understood. It is believed both process utilize similar biological mechanisms, involving activation of extracellular proteases to release tumor cells from the primary site and modify the extracellular matrix at the metastatic sites. Concurrently the tumor cells change the expression profile of its adhesion molecules to adapt to the new microenvironment. This is exemplified by expression of different alpha and beta subunits of integrins displayed by migrating cells ().

Here, we demonstrated a concerted adaptation strategy employed by cancer cells under the regulation of hypoxia and other stress conditions commonly existed in the tumor microenvironment. A novel cysteine endopeptidases legumain is up-regulated in majority of solid tumors. Legumain expression occurs early in both primary and metastatic sites. Normally a lysosomal enzyme, legumain is secreted and functional in the acidic tumor microenvironment. The secretion of legumain is coordinated with the up-regulation of $\alpha v \beta 3$ and $\alpha 5 \beta 1$ integrins. The integrins serve as cell surface receptors as well as co-factors for legumain activity. Binding to integrins enhanced legumain amidolytic activity over hundred fold and is accompanied by an increase of its activity towards its physiologic substrates, such as MMP-2 and cathepsin L. The legumain: integrin proteolytic complex is responsible for activating multiple protease pathways on the invasive cell surfaces. We have strong evidences support that this pericellular protease complex is operative on both tumor and endothelial cells. The legumain: integrin proteolytic complexes represent an distinctive mechanism for MMP2 activation that is not MT-1-MMP and TIMP-2 dependent.

The development of functional vasculatures requires precise spatial-temporal regulation of cell proliferation and differentiation. Legumain activity and localization is regulated by integrins in the tumor microenvironment. Conceivably, legumain can be produced by adjacent cells, such as macrophage, and bind to integrins on nearby cell surfaces. The legumain:integrin complex localizes to the leading edge of invasive cells. Co-localization of legumain with $\alpha\beta 3$ integrin is extensive in human pancreatic cancer supporting the importance of this cell surface proteolytic complex in tumor development.

Addition of recombinant legumain promoted tumor cell migration and drastically accelerated the endothelial cell tube formation on matrigel support. These data further support the functional importance of cell surface legumain:integrin complex in tumor invasion and angiogenesis. It was believed that processing of extracellular matrix and their interaction with integrins account for the formation of tube structure (). This is consistent with legumain activation of MMP2 and cathepsin L and resultant enhanced gelatinase activities that will modify the extracellular matrix to a favorable environment for invasive growth of tumor cells and angiogenesis.

Importantly, the use of high affinity asparaginyl endopeptidase inhibitor (AEPI) to pharmacologically knockout legumain activity has further revealed the role of asparaginyl endopeptidase activity in tumor invasion and angiogenesis. The AEPI demonstrated very low cytotoxicity *in vitro* and lacked gross toxicity *in vivo*, and is very well tolerated.

Our data demonstrated that legumain activity accounts for a majority of enhancement of invasion and angiogenesis induced by hypoxia. AEPI blocks hypoxia induced *in vitro* tube formation in a dose dependent manner. AEPI also inhibited endothelial tube formation under normoxia. More significantly, inhibition of asparaginyl endopeptidase activity pharmacologically with AEPI abolished angiogenesis in *ex vivo* mouse aorta sprouting assay and *in vivo* matrigel angiogenesis assays. AEPI significantly inhibited vessel sprouting as well as vascular extension. These data indicate legumain activity is important to support rapid vessel development under pathologic conditions such as tumor growth. The use of AEPI reduced gelatinase activity locally in tumors presumably through reducing activation of MMP2 and cathepsin L. Both MMP2 and cathepsin L are potent gelatinases that are associated with tumor progression. The hypoxia regulated legumain:integrin complex may initiate the activation of a larger network of protease cascade. These data support indicate legumain:integrin complex is an important proteolytic activator of proteases that are important for angiogenesis, such as MMP2 in addition to the better characterized MT-1-MMP system.

In addition to affecting tumor angiogenesis, suppress legumain activity with AEPI inhibits tumor growth and invasive differentiation of human breast carcinoma cells in nude mouse. The MDA-MB-231 cells form aggressive tumors in nude mice that invade surrounding muscles. The tumors are pathologically similar to human ductal carcinoma

with apparent glandular differentiations. Interestingly the invasive phenotype is reversed by inhibiting legumain activity, even though AEPI has only a modest suppressive effect on primary tumor growth. AEPI-1 treatment resulted in 30% reduction in tumor volume. This may due to the very short half life of our current compound. AEPI-1 activity appears quickly after i.p. injection and peak at 30 minutes post injection. No AEPI activity is detectable with a sensitive legumain activity assay 2 hours after injection. Despite the short half life, AEPI-1 treatment demonstrated significant inhibitory effect on tumor metastasis. Lung metastasis is significantly reduced in 4T1 murine mammary carcinoma in not spontaneous and experimental settings. These data indicate the legumain: integrin proteolytic complex is important breast cancer metastasize to lung. Consistent with this function, tumor cells over-express legumain produce much high level of activated MMP2 versus latent MMP2 and AEPI-1 treatment reduced legumain activity both in vitro and in vivo that are correlated with reduction of active MMP-2 levels.

METHODS

Cells and Reagents

The human MDA-MB231, Panc-1, PC3, HUVEC and mouse 4T1 cell lines are from ATCC. Mouse anti-human Legumain Monoclonal Antibody was purchased from (Abnova). Rabbit anti-human Legumain Polyclonal antibody was affinity purified from rabbit serum which was prepared by immunization with keyhole limpet hemocyanin–conjugated peptide (CGMKRASSPVPLPP). Biotinylated anti-human legumain and Goat anti-human legumain Antibody are purchased from R&D Systems (San Diego, CA). Pro-Legumain were expressed and purified from medium of *Pichia Pastoris*. Auto activation of Legumain is in 50mM citric acid buffer (pH 4.5, containing 1 mM DTT, 1 mM EDTA) for 8hr at 37°C.

Hypoxia Experiments

For experiments that involved hypoxic culture conditions, cells were grown in low-glucose DMEM supplemented 10% heat-inactivated fetal bovine serum. At the initiation of these experiments, the cells were washed twice with PBS. Cells were incubated in either a normoxic environment (standard tissue culture incubator) or in a hypoxic chamber maintained at 1% O₂.

Western Blots

Cells were washed in PBS and lysed in NP-40 buffer (50 mM Tris/HCl, pH 7.4, 250 mM NaCl, 0.5% NP-40, 5 mM EDTA, and 50 mg/ml PMSF). Lysate was cleared by centrifugation, and equal amounts of protein were separated on an 8~16% SDS-Tris/glycine gel. Proteins were transferred to nitrocellulose, and the membrane was blocked with 5% milk powder in TBS plus Tween 20 before probing with antibody to Legumain, Integrin $\alpha_v\beta_3$ (Chemicon), Integrin $\alpha_5\beta_1$ (Chemicon), Cathepsin L(R@D systems) and β -actin(Chemicon).

Flow Cytometry Analysis

Single-cell suspensions of MDA-MB231 were prepared as previously reported (6). Rabbit anti-human Legumain (0.5 µg/mL) was used to detect legumain. This is followed by FITC conjugated goat anti-rabbit IgG diluted 1:5,000 in PBS.

Immunoprecipitations

MDA-MB231 cells were cultured in hypoxic environment for 3 days. After washed 3 times with PBS, cells were lysed in NP-40 buffer. 5µg (anti-integrin α1, α2, α3, αv, αvβ3, β1 and β2 antibody (Chemicon) antibodies to integrin were immunoprecipitated from MDA-MB231 cell lysate (2.4mg/ml) with in per immunoprecipitation reaction separately. Co- immunoprecipitations were also performed by using anti-integrin αvβ3, α5β1 or anti-legumain antibody separately. Irrelevant rabbit or mouse IgG was used as the negative controls.

Histological and Immunohistochemical Analysis

We dissected all kind of Tumor to make frozen section (5 µm) for histological and Immunohistochemical Analysis. Immunohistochemical staining was also performed on Hypoxia culture human MDA-MB231, PC3 and HUVEC cells, which were treated with 10nM EFG in fibronectin coating flask for 3 days. For staining of integrin, mouse monoclonal antibodies to integrin β1, αvβ3 and α5β1(0.1 µg/ml) were used first antibody and Texas-red conjugated anti-mouse IgG (Vector Laboratories) was used as the secondary reporting reagent. For legumain identification, Legumain Polyclonal antibody was used at 0.5 µg/ml, followed by FITC-conjugated anti-Rabbit IgG (Vector Laboratories) as the second antibody. For direct staining of FITC conjugated AEPI-1, The FITC-AEPI-1 was used at 10³ nM and free FITC was a control. F-actin was stained by using Alexa Fluor 647 phalloidin (Invitrogen). The slides were analyzed by using laser scanning confocal microscope (Bio-Rad).

Analyzing the Effect of Integrin αvβ3 Ligation on the Legumain Activity

Fluorometric assay with Z-Ala-Ala-Asn-NHMec was as described with slight modifications (Kembhavi, A, 1993). Varied concentrations of integrin αvβ3 were incubated with activated-legumain(20nM) for 1 hr at 4°C in 0.5 ml assay buffer(50mM citric acid, 121 mM Na₂HPO₄, pH 6.0, containing 1 mM DTT, 1 mM EDTA, and 0.1% CHAPS). Then the substrate (100 µM, in 1.5ml assay buffer) was added to Legumain and αvβ3 mixture. The rate of formation of product was followed in a Perkin-Elmer fluorometer. The excitation and emission wavelengths were 360 and 460 nm, respectively.

Zymogram

The samples were mixed with an equal volume of SDS sample buffer and held at room temperature for 10 min then applied to a zymogram gel (10% Tris-glycine gel with 0.1% gelatin substrate). After electrophoresis, the gel was washed briefly and incubated with 2.5% (v/v) Triton X-100 at room temperature for 30 min with gentle agitation. Digestion of the incorporated gelatin by activated collagenase was conducted in buffer (50 nM Tris (pH7.25), 200 mM NaCl, 10 nM CaCl₂, 0.05% Brij-35 and 0.02% NaN₃) overnight. The

gel was stained with Coomassie Blue R250 (Novex, San Diego, CA), and the presence of a protease was readily observed as a clear band.

Tube Formation Assay

The wells of a 96-well microtiter plate were coated with 60 μ l of ice-cold matrigel and incubated overnight at 37°C. The next day, 12,000 single-donor human HUVEC cells were layered on top of the gel in EGM-2 (Clonetics, San Diego, CA), 2% serum supplemented with 50 ng/ml FGF-2. Each experiment was carried out in triplicate.

Ex vivo Mouse aorta sprouting assay

Mouse aortic ring assays was performed with minor modifications of protocols described previously (Masson V et al, 2002). Mouse aortas were isolated from 4- to 5-week-old female BALB/c nude mice. Isolated aorta was cut into 1-mm square fragments and embedded in 2.2 mg/ml Growth Factor Reduced Matrigel Matrix(400 μ l; Collaborative Biomedical Products, Inc., Bedford, MA) in 24-well plates and cultured in EGM (GIBCO BRL) supplemented with 5% mouse serum and 50 ng/ml FGF-2.

In Vivo Matrigel Plug Assay

This assay was performed as described (Passaniti, A). Growth Factor Reduced Matrigel Matrix (500 μ l; Collaborative Biomedical Products, Inc., Bedford, MA) was mixed with heparin (50 μ g/ml), FGF-2 (400 ng/ml) on ice with or without Legumain inhibitors. The Matrigel mixture was injected into 4–8-week-old female Balb C nu/nu mice at sites near the abdominal midline. Injection sites were chosen such that each animal received a positive control plug (FGF-2 and heparin), a negative control plug (heparin plus buffer), and a plug containing the treatment to be tested (FGF-2, heparin, and AEPI-1). All experiment was carried out in triplicate. Animals were sacrificed 5 days after injection. The mouse skin was detached along the abdominal midline, and the Matrigel plugs were recovered and photographed immediately. Plugs were then dispersed in water and incubated at 37°C overnight. Hemoglobin levels were determined by using Drabkin's solution (Sigma) according to the manufacturer's instructions.

Migration Assays

Cell migration and invasion assays were performed as described with modifications (Albini, A, 1987). The bottom of well was added 500 μ l of medium containing 10% FBS. A suspension of 10^5 cells in 300 μ l of serum-free medium (pH 6.5) was added to the upper chamber. Reagent to be tested, such as Legumain or AEPI-1, was directly added into the cell suspension. The migration assays were carried out at 37°C, 5% CO₂ for 6 hr. At the indicated times, the membrane inserts were removed from the plate, and the non invading cells were removed from the upper surface. Membrane-associated cells were stained with 0.09% crystal violet for 30 min and washed twice with PBS. The invading cells were counted microscopically.

DQ-Collagen Assay

Coat the glass well surface with 50 μ l of Matrigel containing 25 μ g/ml of DQ-collagen IV (30 mm MatTek plates with 14mm well) and leave it in 37°C incubator to solidify for 15

min. Prepare the MDA-MB231 cells: Detach the cells with trypsin, neutralize, spin the cells at 9000 rpm for 4 min. and resuspend the cell pellet at 100,000 ~150,000 cells/ml in serum free medium. Add 2 ml of the cells to each dish. (for better result, use the medium without phenol red. Place in 37°C incubator for 24 to 48 hrs. Observe under microscope and confocal. The fluorescence generated after DQ- collagen IV is hydrolyzed by cellular collagenase. The fluorescence intensity of cells was assayed in a Perkin-Elmer fluorometer.

Mouse Tumor Models

Tumor induction was performed by s.c. injection of 5×10^5 4T1 cells in the right flank of six-week-old BALB/c mice (16). Two different groups of eight animals were treated between day7 after tumor induction. Treatment involved 100ul i.p. injections of either PBS alone (control group) and AEPI-1 treatment group was given 50µg/ 100µl AEPI-1 in PBS by i.p. injection every two days for two weeks. Tumor volumes of treated animals were measured everyday starting on day 6 by micro caliper measurements (volume = width × length × width/2). As soon as the tumor volume reached 1150 mm³ in the control groups (on day 25), euthanasia was performed and lungs were removed and fixed in the Bouin's solution. Lung metastases were counted by anatomy microscope. All of the animal experiments were approved by the Institutional Animal Care and Use Committee of the Scripps research Institute before the experiments were started.

4T1 Lung experimental Metastasis

Six-week-old female BALB/c mice (n=12) were purchased from The Scripps Research Institute animal facilities (San Diego, CA). For i.v. injection, 4T1 cells (1×10^5) were suspended in 0.1 ml sera free medium and injected into the tail vein in mice under the anesthesia. For i.p. Injection, of 5×10^5 4T1 cells in the right flank of balb/c mice. Treatment involved 100µl by i.v. injection of either Saline alone (control group) and AEPI-1 treatment group was given 100µg AEPI-1 in Saline 100ul by i.p. injection daily for two weeks. All mice were euthanized by CO2 and lungs were removed, weighted and fixed in the Bouin's solution. Lung metastases were counted by anatomy microscope.

Vector constructions and retroviral infection

To create Legumain-siRNA retrovirus, the 293 based packaging cells were transfected by Lipofectamine-mediated transfection with 5µg of blank vector or Legumain-siRNA vector construct (Open Biosystems). The medium was changed 6 hr after transfection. At 48 h after transfection, the supernatant was collected and spun at 3000g for 3 min to remove residual cells. Harvest virus in supernatant were transferred to a new tube and filtered through a 0.45-µm filter. MDA-MB-435/GFP Cells were cultured in 60-mm dishes. After the medium was removed, the plates were incubated for 24-48 h with 5 ml of a 1 : 4 mixture of virus supernatants and fresh culture medium containing 0.8µg/ml of polybrene.

MDA-MB-435/GFP Lung experimental Metastasis

MDA-MB-435/GFP cells were injected into the tail lateral vein of female, 4-wk-old Balb/c nude mice (1×10^5 cells/100 μ l of serum free culture medium). Mice were sacrificed after treatment with or without AEPI-1 by i.p. injection daily for 7 days. 3 different cell groups were analyzed: group I (blank vector, 100 μ l Saline), group II (blank vector, 100 μ g AEPI-1/in100 μ l Saline), group III (Legumain-siRNA vector, 100 μ l Saline), 3 mice per group. Lungs were washed in PBS, embedded in OCT and frozen on dry ice. Lungs were evaluated by fluorescence microscopy. Five, 10 μ m sections within 41 thick serial sections (select one section in every ten sections) were examined per Lung.

Statistical analysis

Results are expressed as means \pm s.e.m. Student's paired two-tailed t-test was used to analyze the difference between two groups. Values were regarded significant at $P < 0.05$.

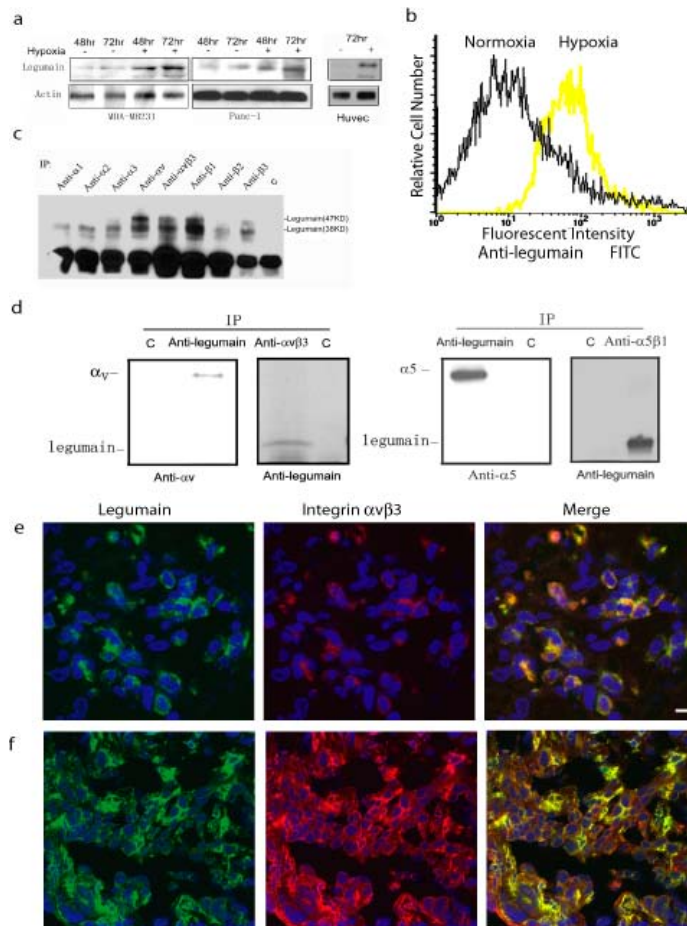


Figure 1 Hypoxia induces Legumain: Integrin protease complex formation. **(a)** Western blot analysis of legumain expression in cells under normoxic and hypoxic conditions. **(b)** FACS analysis of legumain expression on cell surface under normoxic and hypoxic conditions. **(c)** Legumain is immuno-precipitated with a panel of anti-integrin antibodies. **(d)** Immuno-precipitation of legumain by anti $\alpha v \beta 3$ antibody and $\alpha v \beta 3$ by anti-legumain antibody. Same experiment with $\alpha 5 \beta 1$. Immunofluorescence analysis of Legumain: $\alpha v \beta 3$ colocalization in **(e)** MDA-MB231 human breast cancer cells grown in nude mice and **(f)** Panc-1 human pancreatic carcinoma grown in nude mice. The $\alpha v \beta 3$ is red, legumain is green, and cell nuclei are blue. The legumain: $\alpha v \beta 3$ co-localization in complex is yellow. Scale bar, 50 μm .

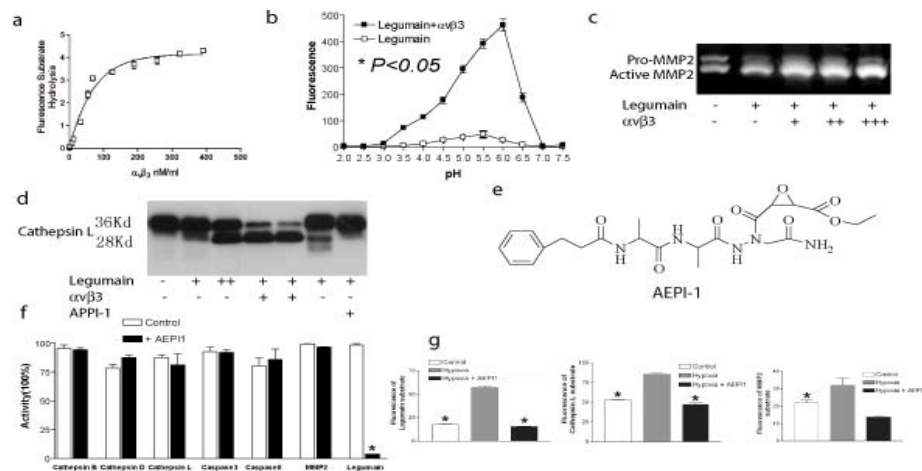


Figure 2 Integrin $\alpha v \beta 3$ is co-factor of legumain activity. **(a)** Binding of $\alpha v \beta 3$ increases the amidolytic activity of legumain in a dose dependent manner. **(b)** Comparison of asparaginyl endopeptidases activity of legumain (clear squares; $n=4$) and legumain: $\alpha v \beta 3$ complex (black squares; $n=4$) in different pH. The $\alpha v \beta 3$ integrin also shift the pH dependency of legumain. **(c)** The $\alpha v \beta 3$ increases its activity towards its physiologic substrate MMP2 as assessed using zymogram, **(d)** cathepsin L by Western blot. **(e)** Asparaginyl endopeptidases inhibitors (AEPI-1). **(f)** AEPI specificity is assessed in activity assay of varied proteases as indicated. AEPI-1 is highly specific to legumain. $* P < 0.001$ versus Legumain control **(g)** Inhibition of legumain activity in tumor cells under hypoxia by AEPI-1 led to decreased activity of MMP-2 and cathepsin L in addition to legumain activity as measured by their specific substrate respectively. $* P < 0.001$ versus Hypoxia, $n=3$. Data represent mean \pm s.e.m.

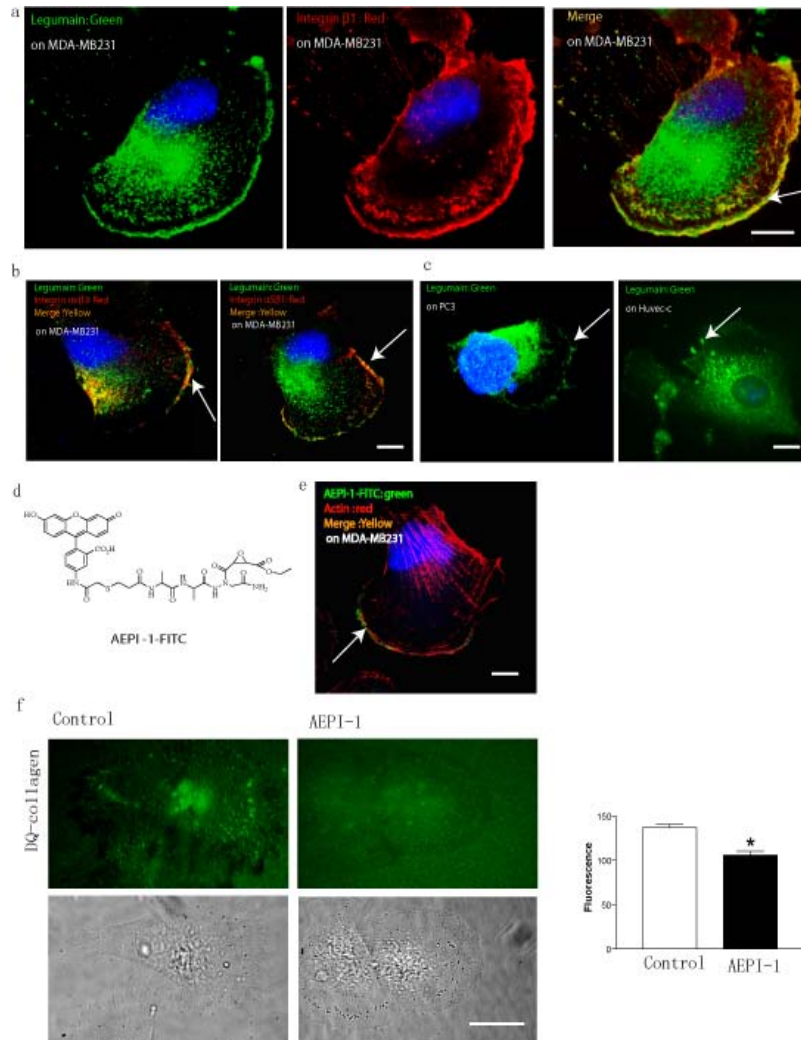


Figure 3. Legumain: integrin regulated matrix remodel at the invasive cell front surface. **(a)** Legumain binds with integrin β_1 exclusively at lamellipodia and invadopodia (white arrow). Scale bar, 20 μ m. **(b)** Legumain binds with integrin $\alpha_v\beta_3$ or $\alpha_5\beta_1$ exclusively at lamellipodia and invadopodia (white arrow). Scale bar, 20 μ m. **(c)** Legumain localization is on cell surface of PC13 and Huvec-c cell (white arrow). Scale bar, 20 μ m. **(d)** Asparaginyl endopeptidases inhibitors (AEPI-1-FITC). **(e)** AEPI-1-FITC attach to MDA-MB231 cell at lamellipodia and invadopodia (white arrow). Scale bar, 20 μ m. **(f)** AEPI-1 inhibit processing of DQ-collagen IV at the invasive cell front surface. Processing of DQ-collagen is quantitated by fluorescence intensity of releasing DQ. Scale bar, 20 μ m. Data represent mean \pm s.e.m.

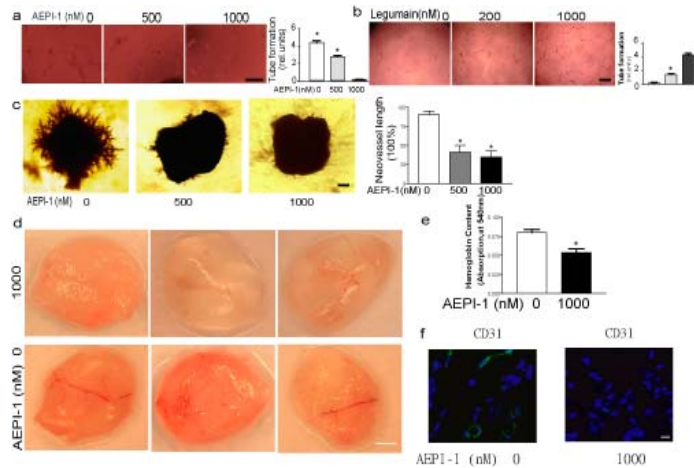


Figure 4 AEPI-1 inhibits angiogenesis in vitro and in vivo. (a) AEPI-1 inhibits HUVEC tube formation in vitro. $*p < 0.05$ versus 1000nM; $n = 4$. Scale bar, 200 μm . (b) Addition of Legumain induces HUVEC tube formation in vitro. Scale bar, 200 μm . $*p < 0.05$ versus 0nM; $n = 4$ (c) AEPI-1 inhibits mouse aorta vessel sprouting assay ex vivo. $*p < 0.001$ versus 0nM; $n = 3$. Scale bar, 500 μm . (d) AEPI-1 inhibits angiogenesis in Matrigel plugs in vivo. Scale bar, 2000 μm . (e) Vessel density is quantitated by hemoglobin content in the plugs. $*p < 0.05$ versus 0nM; $n = 5$ (f) Representative CD31 positive vasculatures in Matrigel plugs. Scale bar, 50 μm . Data represent mean \pm s.e.m.

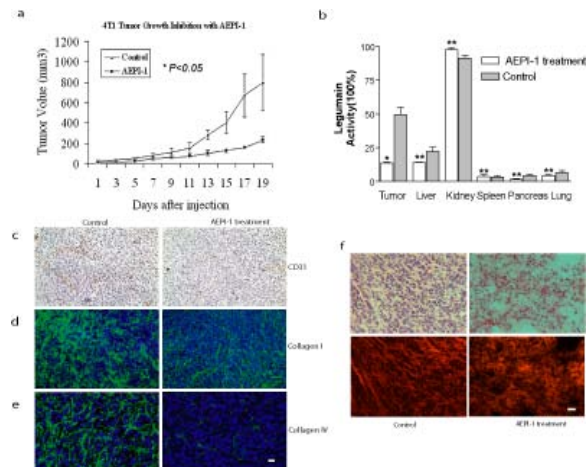


Figure 5 AEPI-1 suppresses matrix remodeling and tumor invasive growth. (a) AEPI -1 inhibits the growth of 4T1 urine mammary carcinoma. $*p < 0.05$ versus 1000nM; $n = 8$ per treatment group. (b) Systemic administration of AEPI-1 inhibited legumain activity in 4T1 tumor, but does not affect legumain activity in kidney, suggesting AEPI-1 is cell impermeable therefore not affecting intracellular legumain. $*p < 0.001$ versus control; $**p > 0.05$ versus control; $n = 4$ (c) AEPI-1 treated tumors have much lower vascular density compare to control tumors. AEPI-1 treated tumors have reduced collagen I (d) and IV (e) content compared to controls. Scale bar, 50 μm . (f) Shape of elastic fiber was affected by AEPI-1 treated tumors. Scale bar, 50 μm . Data represent mean \pm s.e.m.

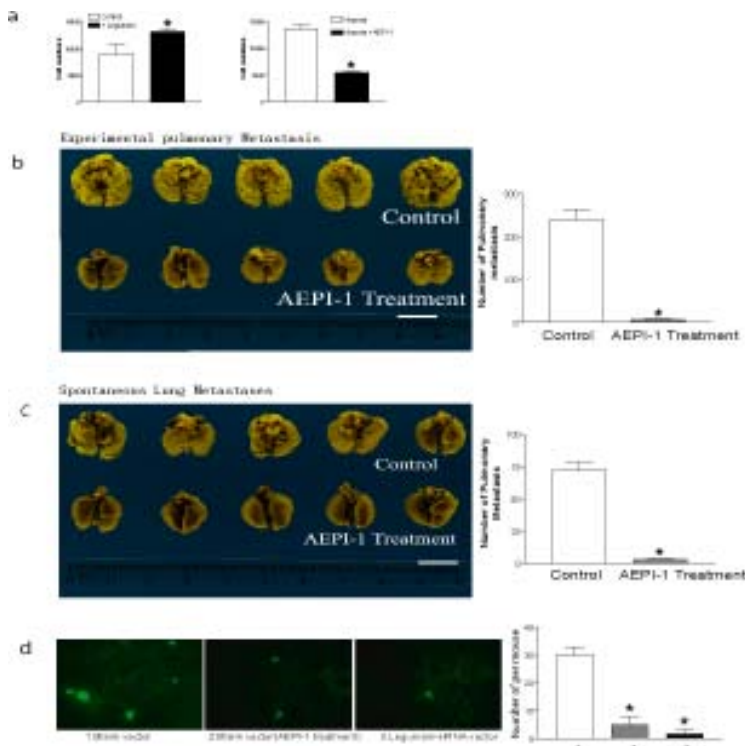


Figure 6 AEPI-1 inhibits distant metastasis. (a) AEPI-1 inhibits experimental lung metastasis of 4T1 mammary carcinoma. * $p < 0.001$ versus control; $n = 6$ per treatment group. Scale bar, 10mm. (b) AEPI-1 inhibits experimental lung metastasis of 4T1 mammary carcinoma. * $p < 0.001$ versus control; * $p < 0.001$ versus control; $n = 6$ per treatment group (c) AEPI-1 inhibits spontaneous lung metastasis of 4T1 mammary carcinoma. * $p < 0.001$ versus control; * $p < 0.001$ versus control; $n = 6$ per treatment group. (d) MDA-MB435-GFP cells metastasize to lung. Scale bar, 10mm. Adenoviral transduction of Legumain SiRNA and Blank vector (Control) cells were quantitated by 5 continued slides of lung tissue. * $p < 0.05$ versus control; $n = 3$ per treatment group. Scale bar, 50 μ m. Data represent mean \pm s.e.m.

Part of the data included in the grant and here was presented at “Cancer, Protease, and the Tumor microenvironment, Nov. 30-Dec. 4, 2005” in an abstract titled “Hypoxia-induced Legumain Expression and Localization to Invasive Cell Surface via Co-factor $\alpha v \beta 3$ Integrins Is Critical for Tumor Invasion and Angiogenesis”.

C. Legubicin action against tumor endothelial cells and tumor associated macrophages. (Months 18-36)

Both primary and metastatic neoplastic development depends on the tumor microenvironment. Tumor growth requires angiogenesis; and the tumor associated macrophage (TAM) is a key producer of an array of growth factors, such as VEGF, to induce angiogenesis and support tumor cell survival. Legumain is an asparaginyl endopeptidase which is specifically over-expressed on the surface of endothelial cells and tumor associated macrophage (TAM) in tumor stroma in addition to the neoplastic

cells. We show that metronomic dosing of LEG-3 effectively kills both tumor associated macrophage and endothelial cells leading to apoptotic death of these cells.

Primary and metastatic neoplastic development both depend on the tumor microenvironment. Tumor growth requires angiogenesis and the tumor associated macrophages (TAMs) are key producers of growth factors, such as VEGF, that induce angiogenesis and support tumor cell survival. Legumain is an asparaginyl endopeptidase which is specifically over-expressed on the surface of endothelial cells and TAM in tumor stroma in addition to the neoplastic cells. Using a doxorubicin-based prodrug specifically activated in the tumor stroma by legumain, we showed that effective killing of both TAMs and endothelial cells. Massive tumor cell death followed the death of TAMs and endothelial cells. The subsequent collapse of tumor micro-vasculatures resulted in complete tumor growth inhibition without any apparent toxicity. Targeting resident cells in the tumor microenvironment has distinctive advantages since both TAMs and endothelial cells are non-transformed and much more sensitive to chemotherapeutic agents versus tumor cells which are frequently multi-drug resistant. The prodrug treatment effectively reduced TAMs in tumors and resulted in significant reduction in angiogenic factors and other growth factors that support tumor cell survival. The anti-angiogenic effect is demonstrated by reduced vessel density in treated tumors. Consequently, the prodrug therapy exerts combined antiangiogenic and anti-tumor effect. The targeted prodrug activation permits metronomic dosing at an effective level critical to prevent the recovery of TAMs and endothelial cells; it effectively seizes the therapeutic windows created by normalization of tumor vasculature following angiogenic factor depletion. Consistent with the importance of TAMs in angiogenesis tumor progression, the accumulation of TAMs proceeds apparent angiogenic responses at the site of metastasis. More importantly, administration of this prodrug significantly reduces metastasis in metastatic models. The prodrug suppresses spontaneous metastasis and significantly reduces circulating tumor cells as demonstrated by blocking metastasis following surgical removal of primary tumors, a clinically relevant setting, and extends survival of the host without toxicity. Our findings indicate TAMs play a critical role in tumor development and metastasis. The potent *in vivo* efficacy suggests that metronomic dosing of legumain-activated prodrug represents a novel anti-cancer strategy targeting multiple steps during tumor metastasis and angiogenesis.

Targeting TAMs or molecules in the tumor microenvironment which attract them and mediate their function represents a novel anti-tumor strategy. TAMs consist of a polarized M2 (CD206+, F4/80+) macrophage population with little cytotoxicity for tumor cells because of their poor production of nitric oxide and proinflammatory cytokines. TAMs also possess poor antigen presenting capacity and effectively suppress T cell activation. In fact, TAMs actually promote tumor cell proliferation and metastases by producing a wide range of growth and pro-angiogenesis factors, metalloproteinases and also partake in circuits that regulate the function of fibroblasts in the tumor stroma.

The selection of Legumain as a target for tumor therapy is based on the fact that the gene encoding this enzyme is highly up-regulated in many murine and human tumor

tissues (Liu, Sun et al. 2003), but absent or only present at very low levels in all normal tissues from which breast tumors arise. Legumain is a distinct member of the C13 family of cysteine proteases (Chen, Dando et al. 1997). It is well conserved throughout the biologic kingdoms, found first in plants, subsequently in parasites, as well as mammals as the only known asparaginyl endopeptidase (AEP). It has a very restricted specificity requiring an asparagine at the P1 site of substrates. In this regard, we recently discovered that Legumain is heavily expressed by TAMs in tumor tissues by using gene expression profiling and immunohistochemistry. Importantly for our studies, TAMs express high levels of legumain in the tumor microenvironment. In contrast, classical macrophages of M1 phenotype, that perform key immune-surveillance functions, do not express Legumain. Consequently, targeting TAMs that over express Legumain does not interfere with the biological functions of normal macrophages, including cytotoxicity and antigen presentation (Sica, Sacconi et al. 2002).

Cell-impermeable Tumor Microenvironment Activated Prodrug (TMEAP) specifically activated only by legumain on the cell surface and target TAMs. We demonstrated that selective killing of Legumain-expressing TAMs down regulate a wide variety of tumor growth factors, pro-angiogenesis factors and enzymes released by these macrophages and lead to inhibition of tumor angiogenesis as well as growth and metastasis. Our data support critical role of TAM in promote tumor cell entering blood stream. This approach may represent a novel cancer therapy against a wide spectrum of solid tumors, since TAM infiltration is evident in high percentage of cancers.

Key Research Accomplishments

1. Demonstrated efficacy of TME activated prodrug, legubicin, against drug resistant breast cancers by killing both tumor and stromal cells such as TAM and endothelial cells.
2. Elucidation of the mechanism of legumain in promoting metastasis/invasion and angiogenesis using multiple approaches including small molecule legumain inhibitor.

Reportable Outcomes

1. Prove of principle of tumor microenvironment activated prodrugs and in vivo demonstration of prodrug efficacy against drug resistant breast cancers (paper published in Cancer Research).
2. The prodrug demonstrated significant anti-cancer efficacy and the data generated from the first year formed a base of a new PCT patent.
3. A new R01 1R01CA127535 "Cysteine Protease Network in Tumor Progression and Therapy" was awarded based on the preliminary results generated from this grant and will continue to support this line of research in future.

4. The Tumor microenvironment activated prodrug approach is licensed for clinical development.

Conclusions

The local tumor microenvironment differs greatly from that of other tissues. One key character is that it is enriched in proteolytic activity. Cell surface proteases, such as legumain, play important role in cancer progression such as invasion/metastasis and angiogenesis. The over-expression of these cell surface proteases are ideal physical as well as functional targets to activate prodrugs in the tumor microenvironment as demonstrated by data generated with the support of this grant.

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Appendices

INHIBITING TUMOR CELL INVASION, METASTASIS AND ANGIOGENESIS

This application claims priority to the filing date of U.S. Provisional Application Ser. No. 60/740,575, filed November 29, 2006, which is incorporated herein by reference in its entirety.

This application is related to PCT Application Ser. No. PCT/US2004/017157 filed May 28, 2004, which claims benefit of U.S. Application Ser. No. 60/474,840 filed May 29, 2003, both of which are incorporated by reference herein in their entireties.

Statement of Government Rights

The invention was made with the support of a grant from the Government of the United States of America (CDMRP Grant Numbers W81XWH-05-1-0091 and W81XWH-05-1-0318 from the Department of Defense). The Government may have certain rights to the invention.

Field of the Invention

The present invention relates to methods for treating and/or inhibiting tumor cell invasion, metastasis and/or angiogenesis as well as increasing apoptosis in cancer cells by administering asparaginyl endopeptidase inhibitors. In some embodiments, the invention relates to inhibitors of proteases that are expressed under the hypoxic conditions of the tumor microenvironment. In other embodiments, the invention relates to prodrug agents that become activated by the proteases that are expressed in the tumor microenvironment. The prodrugs become active within the tumor microenvironments of primary and metastatic tumor sites, for example, at the surface of, cancerous cells and tumor stromal cells that express proteases.

Background of the Invention

The following description includes information that may be useful in understanding the present invention. It is not an admission that any of the information provided herein is prior art, or relevant, to the presently described inventions, or that any publication or document that is specifically or implicitly referenced is prior art.

According to the National Cancer Institute, since 1990 over 17 million people have been diagnosed with cancer, and an additional 1,334,100 new cancer cases are expected to be diagnosed in 2003. About 556,500 Americans are expected to die of cancer in 2003, more than 1500 people every day. Cancer is therefore the second leading cause of death in the United States, exceeded only by heart disease. The National Institutes of Health estimate the overall costs of cancer in the year 2002 at \$171.6 billion (Cancer Facts & Figures, 2003). Clearly, cancer is an enormous problem, and more effective cancer treatments are needed.

Two characteristic features of malignant cells are the ability to invade normal tissues and the ability to spread to distant sites. Tumor metastasis and invasion are the main cause of cancer mortality. Malignant cells can

spread by several routes including direct local invasion, by the lymphatics or by capillaries. Local invasion is accomplished by an increase of tumor cell mobility and by production of proteases that destroy the normal extracellular matrix and basement membranes. Once the tumor cells escape from their normal boundaries, they are free to enter the circulation through the capillaries and the lymphatic system. The need for methods to prevent tumor invasion and metastasis is critical and constitutes a major goal in the effort to develop effective therapeutic interventions against cancer.

In addition, many cancer cells are capable of inducing angiogenesis. To form blood vessels, angiogenic endothelial cells share some of the same biochemical mechanisms that are used by cancer cells to invade local tissues.

Current cancer treatments generally involve the use of surgery, radiation therapy, and/or chemotherapy. However, these treatments all involve serious side effects. For example, surgery can be complicated by bleeding, damage to internal organs, adverse reactions to anesthesia or other medicines, pain, infection, and slow recovery. Radiation therapy can damage normal cells and can cause fatigue. For many people, chemotherapy is the best option for controlling their cancer. However, chemotherapy can also damage normal cells such as bone marrow and blood cells, cells of the hair follicles, and cells of the reproductive and digestive tracts. Chemotherapy can also cause nausea, vomiting, constipation, diarrhea, fatigue, changes to the nervous system, cognitive changes, lung damage, reproductive and sexual problems, liver, kidney, and urinary system damage, and, especially with the use of the chemotherapeutic agent doxorubicin, heart damage. Long-term side effects of chemotherapy can include permanent organ damage, delayed development in children, nerve damage, and blood in the urine. Thus, the use of the chemotherapy for cancer treatment is not without serious side effects.

Most agents currently administered to a patient are not targeted to the site where they are needed, resulting in systemic delivery of the agent to cells and tissues of the body where the agent is unnecessary, and often undesirable. Such systemic delivery may result in adverse side effects, and often limits the dose of an agent (*e.g.*, cytotoxic agents and other anti-cancer agents) that can be administered. Accordingly, a need exists for agents and methods that specifically target cancerous cells and tissues.

Thus, it would be desirable to be able to direct various agents to cancer cells and to the tumor microenvironment so as to be able to decrease the dosage of the agents given and to decrease the systemic toxicity and side effects associated with these agents.

Summary of the Invention

According to the invention, the tumor microenvironment creates conditions that induce expression of certain genes, including proteases that are active almost exclusively in the tumor microenvironment. For example, an unexpectedly high level expression of asparaginyl endopeptidases, including legumain, is present in a wide variety of cancer cells, particularly those involved in metastasis. Other proteases that are active in the tumor microenvironment include prostate specific membrane antigen (PSMA)(a carboxypeptidase), fibroblast activation protein (FAP) (a serine peptidase), cathepsin B (a cysteine protease), cathepsin X (a cysteine protease), urokinase-

type plasminogen activator (uPA)(a serine protease), tissue factor VIIa (TF VIIa)(a serine protease), matriptase (a membrane-bound serine protease) and Factor XIII. As described herein, tumor-specific protease expression occurs early in the development of cancer cell invasion, just as metastasis begins, and under the hypoxic conditions associated with invasive tumor growth. Moreover, legumain is directly involved in and actually inhibits the cascade of activities that leads to cellular apoptosis, particularly in cancer cells where legumain is highly expressed.

The tumor specific proteases described herein are cell surface associated proteases. These proteases function in protease networks that play critical roles in modulating extracellular matrix proteins. For instance, certain cancers may employ more cysteine proteases than metalloproteinases or serine protease and vice versa. However the matrix modifying function of each of these proteases is indispensable for tumor metastasis and invasive growth.

According to the invention, the combined use of protease activity imaging agents, protease inhibitors, and/or prodrugs described herein represents an integrated precision-guided cancer therapeutic system. The combined use of protease inhibitor and prodrugs are also envisioned, although in some embodiments the prodrug and protease inhibitor targeting same protease are used at different times during the therapeutic regimen.

Also as shown herein, legumain activity is substantially increased on the surface of tumor cells by cell-surface association with integrins, indicating that integrins are co-factors for legumain. Moreover, legumain can activate metalloproteinases (e.g. MMP-2) and cathepsins (e.g., cathepsins B, H and L), which are all proteases involved in promoting tumor cell invasion and metastasis. In addition, asparaginyl endopeptidase expression is also associated with reduced cancer cell apoptosis and increased angiogenesis. Therefore, expression of, and activity by, certain proteases, including legumain, PSMA, FAP, cathepsin B, cathepsin X, uPA, tissue factor VIIa, matriptase (a membrane-bound serine protease) and Factor XIII are cancer and angiogenesis markers and constitute indicia of tumor cell metastasis. The invention therefore provides agents to treat undesirable angiogenesis, tumor cell invasion, tumor cell metastasis and other such cancerous conditions, particularly those conditions involving cells and tissues that express these proteases.

Many tumor cells are largely resistant to chemotherapy, for example, because the chemotherapeutic agents employed are only active against a subset of the tumor cells that comprise a cancerous condition. According to the invention, stromal cells in the tumor microenvironment, such as endothelial cells or tumor associated macrophages (TAMs), can be targeted by the agents of the invention to effectively treat these drug-resistant tumor cell types. This strategy is also effective for reducing the expression and/or activity of molecules in the tumor microenvironment that attract TAMs and other tumor-associated cells that facilitate tumor growth and invasion. TAMs consist of a polarized M2 (CD206+, F4/80+) macrophage population. TAMs also possess poor antigen presenting capacity and effectively suppress T cell activation. In fact, TAMs actually promote tumor cell proliferation and metastases by producing a wide range of growth factors, pro-angiogenesis factors, metalloproteinases and the like. TAMs also partake in circuits that regulate the function of fibroblasts in the tumor stroma and are particularly abundantly expressed in the tumor stroma.

According to the invention, TAMs express high levels of certain proteases, including legumain, in the tumor microenvironment. In contrast, classical macrophages of the M1 phenotype, that perform key immune-surveillance functions, do not express legumain. Consequently, targeted elimination of TAMs does not interfere with the biological functions of normal (M1) macrophages, including cytotoxicity and antigen presentation. Thus, one aspect of the invention involves targeting legumain-expressing TAMs with prodrugs and/or protease inhibitors to destroy TAMs and/or inhibit their function.

TAM and endothelial cells are non-transformed cells therefore will not develop drug resistance that is common among malignant cancers. Thus, low dosages of the prodrugs and/or protease inhibitors can be employed when targeting these TAM and endothelial cells. This will down regulate a wide variety of tumor growth factors, pro-angiogenesis factors and enzymes released by these macrophages and lead to inhibition of tumor angiogenesis as well as invasive growth and metastasis.

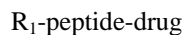
One aspect of the invention is a method of treating cancer in a mammal by administering to the mammal an effective amount of a prodrug or an inhibitor of a protease that is expressed in the tumor microenvironment. According to the invention, treatment of cancer can involve killing tumor cells, reducing the growth of tumor cells and reducing the growth or function of tumor stromal cells in a mammal. Examples of stromal cells that can be treated by the methods of the invention include tumor-associated macrophages and endothelial cells. Treatment of cancer can also involve promoting apoptosis of cells that express legumain. As shown herein, expression of legumain inhibits apoptosis and cancer cells that exhibit high levels of legumain expression resist apoptosis. Treatment of cancer can also involve inhibiting angiogenesis of a tumor in a mammal.

Another aspect of the invention is a protease-activated prodrug that is tumoricidal *in vivo*, wherein the protease is a protease that is expressed in the tumor microenvironment (e.g., under the hypoxic conditions of tumor microenvironments). These protease-activated prodrugs have reduced side effects and toxicity relative to currently available chemotherapeutics. While the present prodrugs are useful for treating cancer, they are also useful to treat other conditions and cellular environments that express proteases. For example, certain non-transformed cells support tumor growth and invasion and, as described herein also express proteases when present in the tumor microenvironment. Hence, the present prodrugs can be used to target and kill not only cancer cells but also the cells that support tumor growth and tumor cell metastasis.

A prodrug compound of the invention includes a drug molecule linked to a peptide, wherein the peptide has an amino acid sequence that is specifically recognized by a protease expressed in a tumor microenvironment. Thus, the peptide includes at least two linked amino acids, wherein at least one of the two linked amino acids is an amino acid that is specifically recognized by a tumor-specific protease and forms part of a cleavage site for the protease. For example, legumain is a protease that is specifically expressed in tumor cells and cells that support tumor growth and metastasis (e.g. tumor associated macrophages). Legumain is an asparaginyl protease that specifically recognizes asparagine-containing peptides and cleaves peptides that contain asparagine (Asn). Some of the prodrugs of the invention are therefore designed to be activated by legumain. Legumain cleaves the peptide of the present prodrugs at the site of the Asn to generate an active drug from the prodrug. Prior to cleavage, the prodrug is

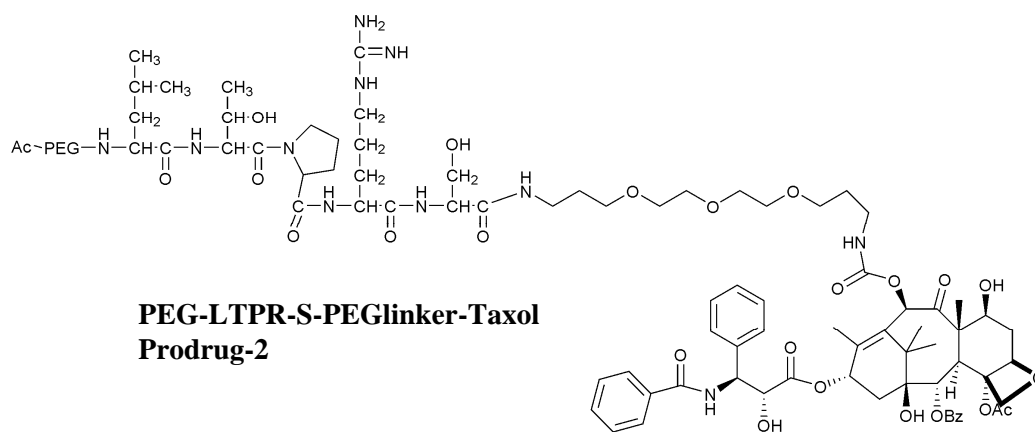
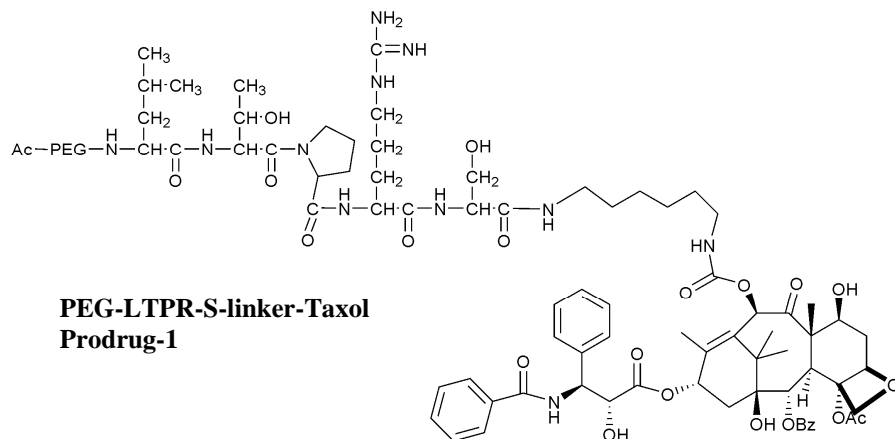
substantially non-toxic to normal animal cells, whereas after cleavage, the drug is an active drug that can have a beneficial effect upon an animal to which it is administered.

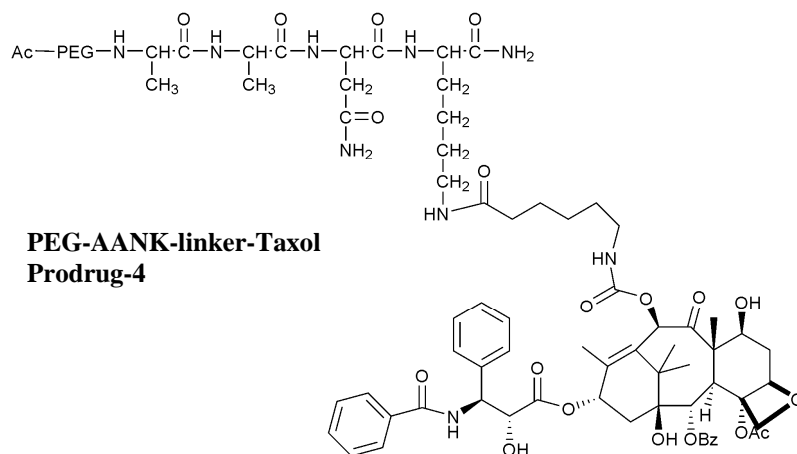
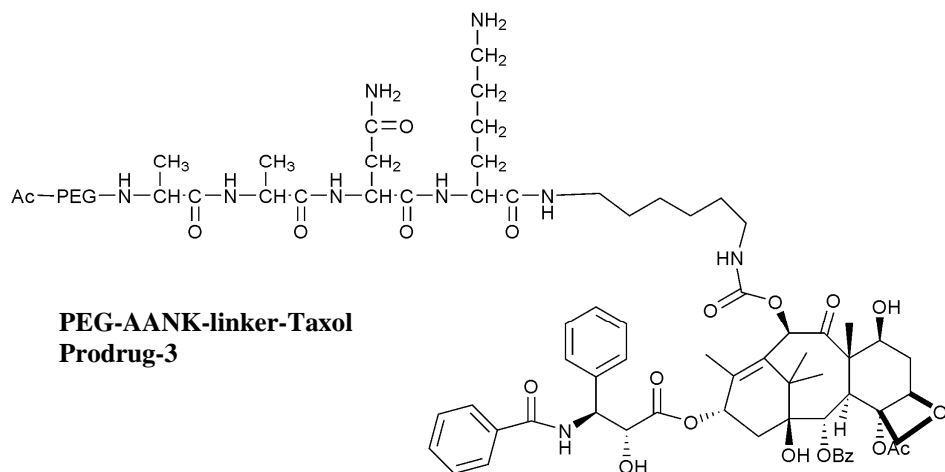
Prodrugs of the invention have the general structure:



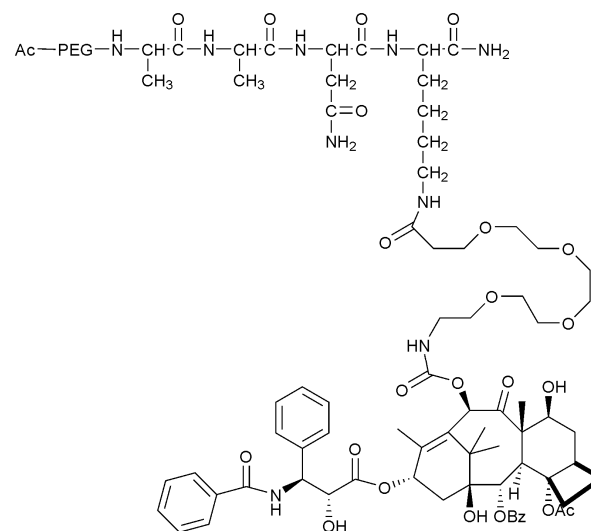
wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group; and drug is any therapeutic agent. In some embodiments, the R_1 groups of the present prodrugs are not hydrophobic groups or labels. The R_1 and drug moieties can be linked directly to the peptide or they can be linked to the peptide through a linker or spacer molecule. Such a linker or spacer can be an alkylene, a sugar or an oligosaccharide.

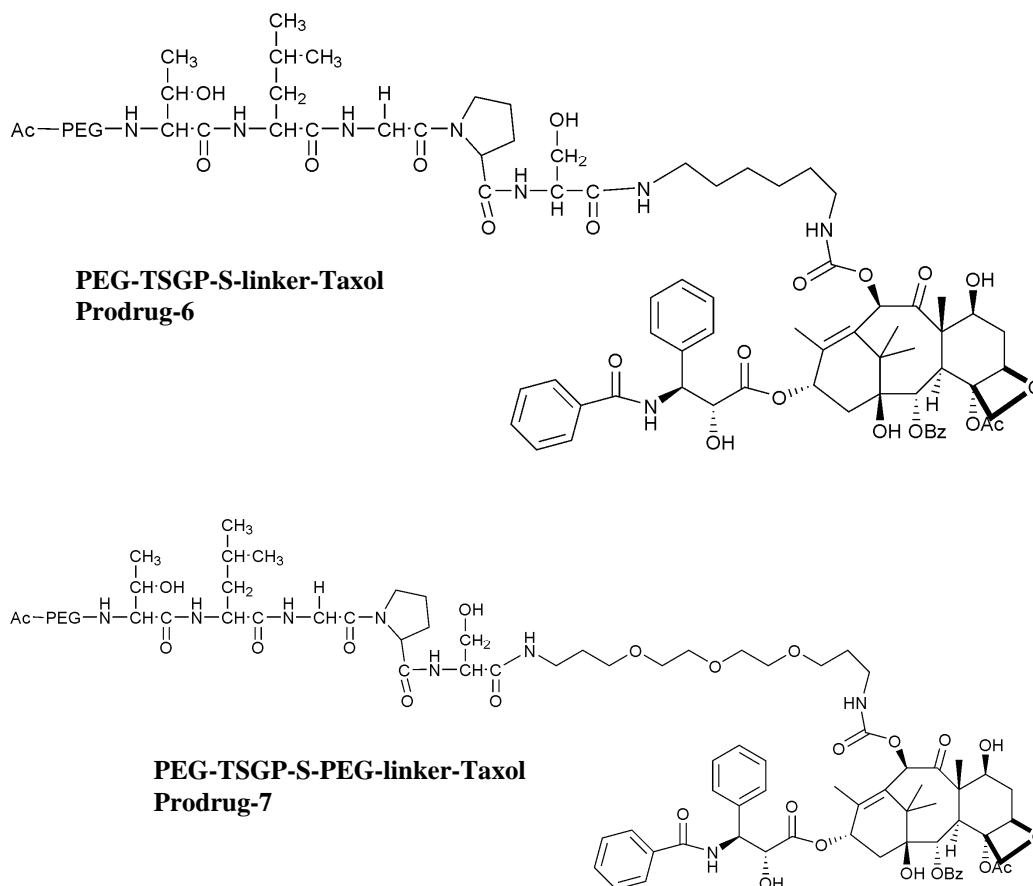
Specific examples of prodrug compounds of the invention include, for example, taxol, paclitaxel, doxorubicin containing prodrugs, including those shown below:





PEG-AANK-PEGlinker-Taxol (Prodrug-5):





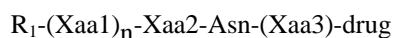
Prodrug-1 and prodrug-2 are activated by serine protease TF VIIa. Prodrug-3, prodrug-4 and prodrug-5 are activated by legumain. Additional legumain prodrugs include LEG-2 (*N*-Succinyl- β -alanyl-L-threoinyl-L-Asparaginyl-L-Leucyl-Doxorubicin) and LEG-3 (*N*-Succinyl- β -alanyl-L-alanyl-L-Asparaginyl-L-Leucyl-Doxorubicin), whose structures are shown in FIG. 9A. In some instances, the prodrugs of the invention do not include LEG-2 or LEG-3, because the inventor has filed a separate application on these compounds. Prodrug-6 and prodrug-7 are activated by fibroblast activation protein (FAP).

The drug employed is any drug whose action is diminished or blocked by attachment of a peptide to the drug. The ability of the drug to enter cells is diminished, inhibited or blocked by attachment of the peptide and hydrophilic groups. Such hydrophilic groups are generally included to facilitate water-solubility and cell impermeability. Hydrophilic groups are generally attached to the peptide so that the function of the drug is not inhibited or blocked by the hydrophilic group once the peptide is cleaved from the prodrug to yield the drug.

In some embodiments, the drug can be a cytotoxin or a photosensitizing agent. Such a cytotoxin can be aldesleukin, 5-aminolevulinic acid, bleomycin sulfate, camptothecin, carboplatin, carmustine, cisplatin, cladribine, lyophilized cyclophosphamide, non-lyophilized cyclophosphamide, cytarabine, dacarbazine, dactinomycin, daunorubicin, diethylstilbestrol, epoetin alfa, esperamycin, etidronate, etoposide, filgrastim, floxuridine, fludarabine phosphate, fluorouracil, goserelin, granisetron hydrochloride, idarubicin, ifosfamide, immune globulin, interferon alpha-2a, interferon alpha-2b, leucovorin calcium, leuprolide, levamisole, mechlorethamine, medroxyprogesterone,

melphalan, methotrexate, mitomycin, mitoxantrone, octreotide, ondansetron hydrochloride, paclitaxel, pamidronate, pegaspargase, plicamycin, protoporphyrin IX, sargramostim, streptozocin, taxol, thiotepa, teniposide, vinblastine, or vincristine. In some embodiments, the drug is doxorubicin, 5-aminolevulinic acid, protoporphyrin IX, taxol or paclitaxel.

In one embodiment, the prodrug is activated by asparaginyl proteases (e.g., legumain) and has a peptide amino acid sequence comprising SEQ ID NO:3:



wherein:

R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group;

n is an integer of about 0 to about 50;

Xaa1 and Xaa2 are separately any amino acid;

Xaa3 is either nothing or an amino acid that has no substantial effect on the activity of the drug;

and

the drug employed is a drug whose action is diminished or blocked by attachment of a peptide to the drug.

In some embodiments, the R_1 groups of the present prodrugs are not hydrophobic groups or labels. For example, when cytotoxic drugs are part of the prodrug, a hydrophilic group is preferably used for R_1 to limit cell uptake by non-target cells.

Examples of peptide sequences that may be used in the prodrugs of the invention include amino acid sequence Asn-Leu, Ala-Asn-Leu, Thr-Asn-Leu, Ala-Ala-Asn-Leu (SEQ ID NO:5), Ala-Thr-Asn-Leu (SEQ ID NO:6), and Boc-Ala-Ala-Asn-Leu (SEQ ID NO:4). Examples of prodrugs provided by the invention include Boc-Ala-Ala-Asn-Leu-doxorubicin (SEQ ID NO:7), succinyl-Ala-Ala-Asn-Leu-doxorubicin (SEQ ID NO:8), N-(-*t*-Butoxycarbonyl-Ala-Thr-Asn-Leu)doxorubicin (SEQ ID NO:9), N-(Succinyl-Ala-Thr-Asn-Leu)doxorubicin (SEQ ID NO:10), N-(-*t*-Butoxycarbonyl-Ala-Asn-Leu)doxorubicin (SEQ ID NO:11), N-(Succinyl-Ala-Asn-Leu)doxorubicin (SEQ ID NO:12), N-(-*t*-Butoxycarbonyl-Thr-Leu)doxorubicin (SEQ ID NO:13), N-(Succinyl-Thr-Leu)doxorubicin (SEQ ID NO:14),

As described herein a hydrophilic R_1 group (sometimes abbreviated herein as "Hyd") facilitates prodrug and inhibitor water solubility and inhibits cell uptake and tissue retention of the prodrug before activation and of the inhibitor before binding to a protease (e.g. the legumain: integrin complex). A variety of hydrophilic protecting groups can be utilized. Hydrophilic R_1 groups of the invention can be sugars (monosaccharides and disaccharides), dicarboxylic acids (e.g., succinate, malate, fumarate, oxaloacetate, citrate, isocitrate), glycans, polyalkylene oxides, lower alkyl carboxylates, carboxyalkyls, carboxyalkylene carboxylates, charged amino acids (e.g., any of the hydrophilic, acidic, basic and polar amino acids described herein) and the like. In some embodiments the protecting group is a hydrophilic amino protecting group. Specific examples of R_1 groups that can be used include glucuronide,

succinyl, polyethylene glycol (PEG) or glutathione. Hydrophobic groups can be used if cellular uptake of the drug is desired. Hydrophobic groups that can be used include those listed herein.

The invention also provides a protease inhibitor having including formulae **III**, **IV**, **V** or **VI**:



wherein:

wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group;

n is an integer of about 2 to about 5;

each Xaa4 and Xaa5 is an amino acid or an amino acid mimetic;

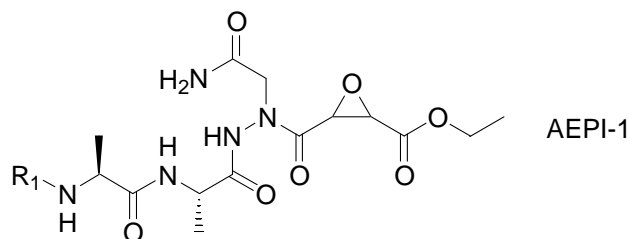
Y is alkyl, alkenyl epoxide, fluoromethylketone or a Michael acceptor, optionally substituted with 1-3 halo or hydroxy, alkylamino, dialkylamino, alkyldialkylamino, or cycloalkyl, alkylcycloalkyl, alkenylcycloalkyl, aryl; (C_5-C_{12}) arylalkyl or (C_5-C_{12}) arylalkenyl,

wherein the aryl groups of the arylalkyl or arylalkenyl can be 0-4 heteroatoms selected from N, O and S, and are optionally substituted with halo, cyano, nitro, haloalkyl, amino, aminoalkyl, dialkylamino, alkyl, alkenyl, alkynyl, alkoxy, haloalkoxy, carboxyl, carboalkoxy, alkylcarboxamide, (C_5-C_6) aryl, $-O-(C_5-C_6)$ aryl, arylcarboxamide, alkylthio or haloalkylthio; and

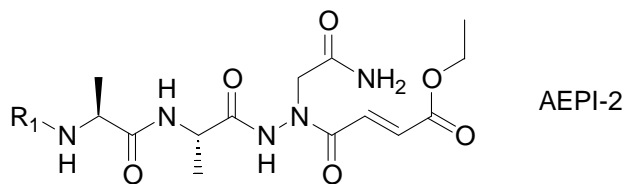
wherein each of the inhibitors of formulae **III**, **IV**, **V** and **VI** bind to a protease expressed in a tumor microenvironment.

Examples of asparaginyl endopeptidase inhibitors (AEPIs) that may be used in the methods of the invention include the following:

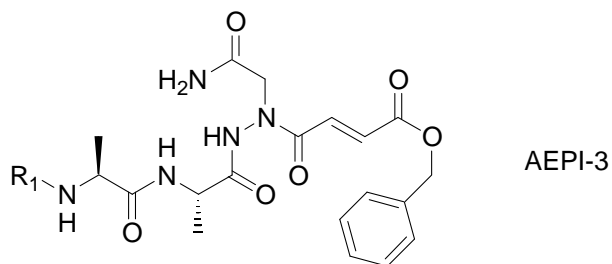
AEPI-1 is R_1 -Ala-Ala-AzaAsn-(S,S)-EPCOOEt, a compound of the structure:



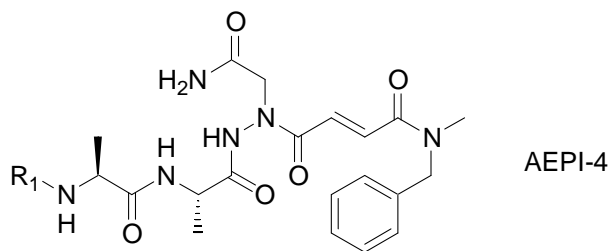
AEPI-2 is R_1 -Ala-Ala-AzaAsn-CH=CH-COOEt, for example, a compound of the structure:



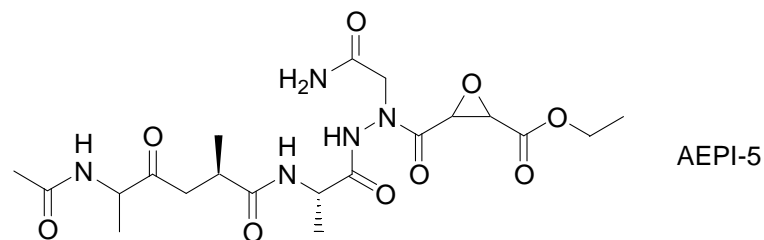
AEPI-3 is R_1 -Ala-Ala-AzaAsn-CH=CH-COOBzl, for example, a compound of the structure:



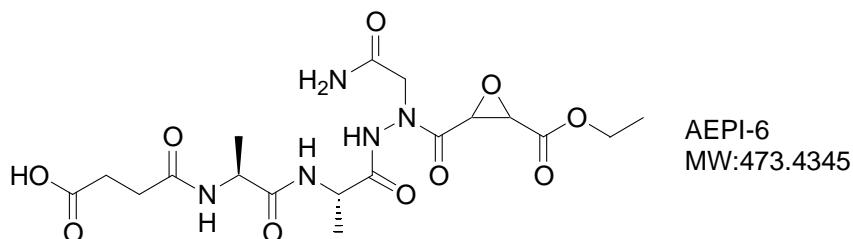
AEPI-4 is R_1 -Ala-Ala-AzaAsn-CH=CH-CON(CH₃)Bzl, for example, a compound of the structure:



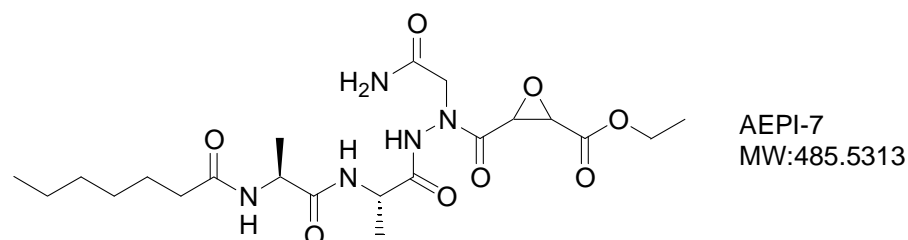
AEPI-5 is N-acetyl-Ala-Ala-AzaAsn-(S,S)-EPCOOEt, a compound of the structure:



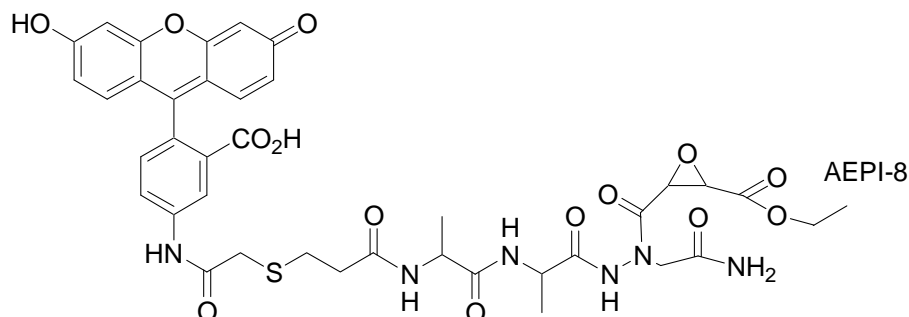
AEPI-6 is N-succinyl-Ala-Ala-AzaAsn-(S,S)-EPCOOEt, a compound of the structure:



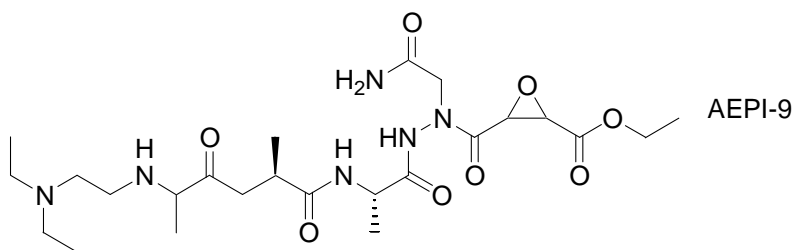
AEPI-7 is N-heptanoyl-Ala-Ala-AzaAsn-(S,S)-EPCOOEt, a compound of the structure:



AEPI-8 is 3-(N-carbamoylmethyl-N'-{fluorescein]-methylsulfanyl}-N-propionyl)-Ala-Ala-AzaAsn-(S,S)-EPCOOEt, a compound of the structure:

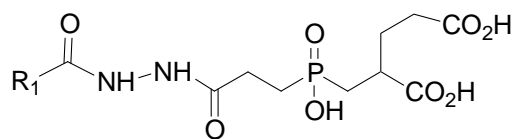


AEPI-9 is N-triethylamino-Ala-(N-isopropanoyl-Ala-AzaAsn-(S,S)-EPCOOEt), a compound of the structure:



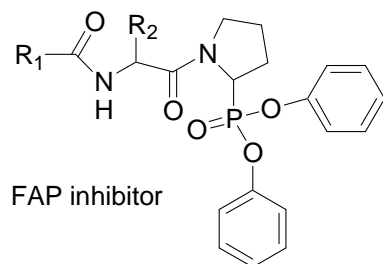
wherein R₁ is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group; Ep is epoxy; Et is ethyl; and Bzl is benzyl.

Inhibitors of PSMA that can be used in the invention include the following:



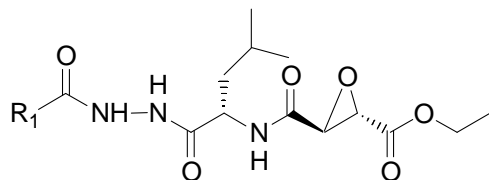
wherein R₁ is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group.

Inhibitors of fibroblast activation protein (FAP) that can be used in the invention include the following:



wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group; and R_2 is hydrogen, hydroxymethylene (CH_2OH), lower alkyl (e.g., methyl, ethyl, propyl, isopropyl ($\text{CH}(\text{CH}_3)_2$), butyl, isobutyl), or benzyl.

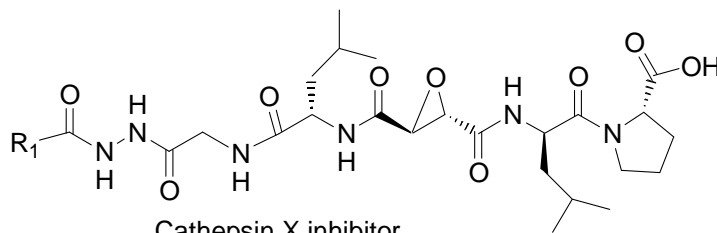
Cathepsin B inhibitors useful in the invention include the following:



Cathepsin B inhibitor

wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group.

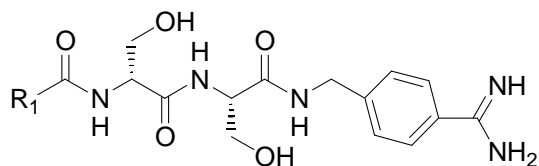
Cathepsin X inhibitors useful in the invention include the following:



Cathepsin X inhibitor

wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group.

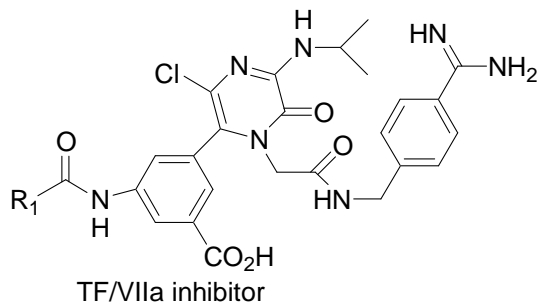
Urokinase-type plasminogen activator (uPA) inhibitors useful in the invention include the following:



uPA inhibitors

wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group.

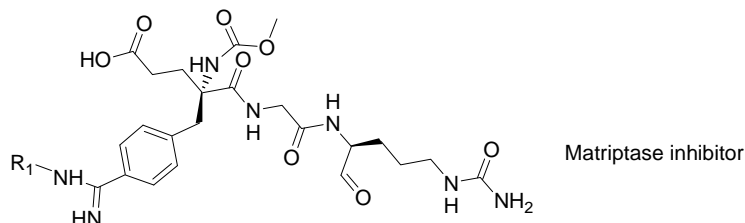
Tissue factor VIIa (TF VIIa) inhibitors useful in the invention include the following:



TF/VIIa inhibitor

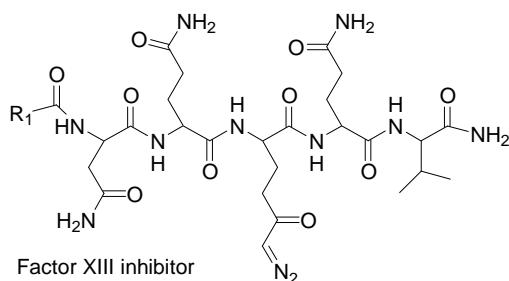
wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group.

Matriptase inhibitors useful in the invention include the following.



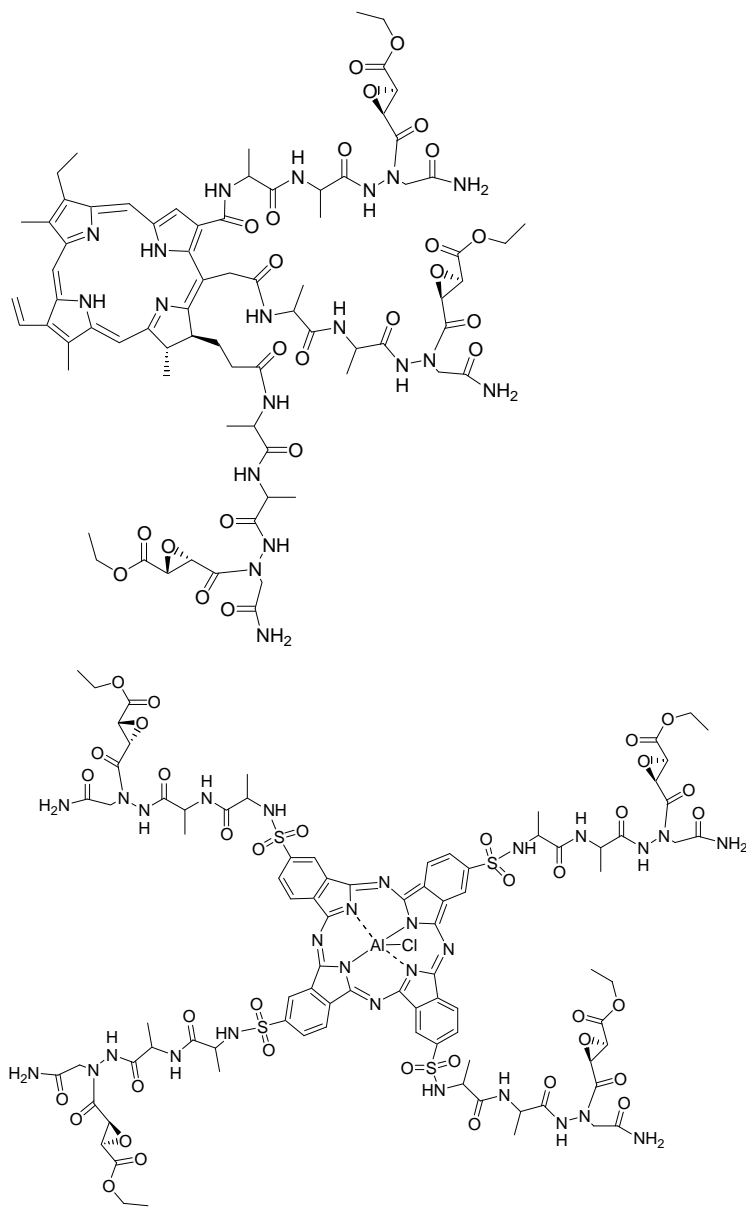
wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group.

Factor XIII inhibitors useful in the invention include the following.



wherein R_1 is hydrogen, a hydrophilic group, a hydrophobic group, a photosensitizing agent, a label or an amino protecting group.

In some embodiments, the inhibitor is linked to a photosensitizing agent. While any available photosensitizing agent can be used (e.g., any of the photosensitizing agents contemplated for use with the present prodrugs), specific examples of photosensitizing agents include chlorin e6 and aluminum phthalocyanine tetrasulfonate (AlPcS4). Examples of inhibitors with chlorin e6 and AlPcS4 are shown below.



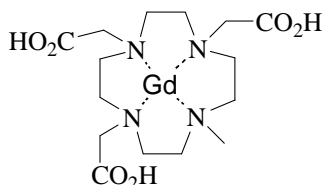
Hydrophilic R_1 groups of the invention can be sugars (monosaccharides and disaccharides), dicarboxylic acids (e.g., succinate, malate, fumarate, oxaloacetate, citrate, isocitrate), glycans, polyalkylene oxides, lower alkyl carboxylates, carboxyalkyls, carboxyalkylene carboxylates, and the like.

Hydrophobic R_1 groups of the invention can be alkyl, aryl, alkylene aryl, arylalkyl, hydrophobic amino protecting agents, beta-alanyl and related hydrophobic groups.

Any convenient amino protecting group available in the art can be used in the invention including, for example, carbobenzyloxy (Cbz), *tert*-butyloxycarbonyl (BOC), 9-fluorenylmethyloxycarbonyl (Fmoc) and benzyl groups.

A variety of labels can be used with the inhibitors of the invention to generate imaging agents or reagents for detection of cancer. Such labels can be fluorophores, radioisotopes, metals, enzymes, enzyme substrates,

luminescent moieties, and the like. One example of a label that may be used is gadolinium or a gadolinium complex. For example, the following gadolinium complex can be used as a label:



In other embodiments, the legumain inhibitor can be cystatin, stefin, a peptide including the sequence Ala-Leu-β-Asn-Ala-Ala (SEQ ID NO:15) or an antibody that inhibits legumain activity.

Another aspect of the invention is a pharmaceutical composition that includes at least one of the prodrug compounds of the invention or at least one of the protease inhibitors of the invention and a pharmaceutically acceptable carrier. In some embodiments, the carrier is a liposome. Combinations of the present prodrug compounds and/or protease inhibitors can also be included in the compositions of the invention.

Another aspect of the invention is a method of detecting and treating cancer in a mammal, comprising administering to the mammal an inhibitor of the invention, wherein R₁ is a label, to detect whether the mammal has cancer and to detect which type of tumor specific protease is associated with the cancer, and administering a prodrug of the invention to treat the cancer, wherein the prodrug has a cleavage site for the tumor specific protease associated with the cancer.

Another aspect of the invention is a method for treating a mammal having, or suspected of having cancer. The method includes administering to the mammal a prodrug compound and/or a protease inhibitor of the invention in amounts and at intervals effective to prevent, reduce, or eliminate one or more of the symptoms associated with cancer. The cancer can be an invasive or metastatic cancer. The cancer can also be a tumor that is prone to angiogenesis. Cancers that can be treated by the invention include solid tumors and cancers as well as cancers associated with particular tissues, including breast cancer, colon cancer, lung cancer, prostate cancer, ovarian cancer, cancer of the central nervous system, carcinomas, leukemias, lymphomas, melanomas, fibrosarcomas, neuroblastoma, and the like. The cancer can, for example, be autoimmune deficiency syndrome-associated Kaposi's sarcoma, cancer of the adrenal cortex, cancer of the cervix, cancer of the endometrium, cancer of the esophagus, cancer of the head and neck, cancer of the liver, cancer of the pancreas, cancer of the prostate, cancer of the thymus, carcinoid tumors, chronic lymphocytic leukemia, Ewing's sarcoma, gestational trophoblastic tumors, hepatoblastoma, multiple myeloma, non-small cell lung cancer, retinoblastoma, or tumors in the ovaries.

The invention also provides a method for inhibiting cancer metastasis and/or tumor cell invasion in an animal, including administering a protease inhibitor compound and/or prodrug of the invention to the animal in amounts and at intervals effective to prevent, reduce, or eliminate cancer metastasis and/or tumor cell invasion.

The invention also provides a method for inhibiting cell migration in an animal that includes administering a protease inhibitor compound and/or prodrug of the invention to the animal in amounts and at intervals effective to prevent, reduce, or eliminate cancer cell migration.

The invention also provides a method of killing a cell in a tissue, including contacting the cell with a prodrug of the invention in amounts and at intervals effective to kill the cell, wherein the tissue includes cells that express legumain.

The invention also provides a method for treating cancer in animal that includes administering to the animal a protease inhibitor compound or a prodrug of the invention in amounts and at intervals effective to prevent, reduce, or eliminate one or more symptoms of cancer in the animal.

The invention also provides a method for inhibiting cancer metastasis in a tissue that includes contacting the tissue with a protease inhibitor compound or prodrug of the invention in amounts and at intervals effective to prevent, reduce, or eliminate cancer metastasis.

The invention also provides a method for inhibiting cancer cell migration in a tissue that includes contacting the tissue with a protease inhibitor compound or prodrug of the invention in amounts and at intervals effective to prevent, reduce, or eliminate cancer cell migration.

The invention also provides a method for treating inflammation in an animal, which includes administering to the mammal a protease inhibitor compound or prodrug of the invention in amounts and at intervals effective to prevent, reduce, or eliminate one or more symptoms associated with inflammation.

The invention also provides a method for delivering a drug to a cell in a tumor microenvironment of a mammal, which includes administering to the mammal an effective amount of a prodrug of the invention.

The invention also provides a method for diagnosing cancer in a tissue that includes contacting the tissue with of an agent that specifically binds to a protease that is expressed in a tumor microenvironment, and detecting whether the agent binds to the tissue. The invention also provides a method for diagnosing cancer in an animal that includes administering to the animal an agent that specifically binds to a protease that is expressed in a tumor microenvironment, and detecting whether the agent accumulates in a tissue. For example, the protease can be selected from the group legumain, PSMA, FAP, Cathepsin B, Cathepsin X, uPA, TF VIIa, matriptase and Factor XIII. These methods can further include diagnosing the patient as having or not having cancer and monitoring the progression of a cancer.

The invention also provides a method for imaging a tissue that expresses a protease. For example, the protease can be selected from the group legumain, PSMA, FAP, Cathepsin B, Cathepsin X, uPA, TF VIIa, matriptase and Factor XII. The method includes contacting a test tissue suspected of expressing the protease with of an agent that specifically binds to the protease, and detecting whether the agent binds to the test tissue. The method can further include quantifying and comparing amounts of the agent bound to the test tissue with amounts of the agent bound to a control tissue that does not express the protease. The agent can be protease inhibitor-imaging agent or a labeled antibody that specifically binds to the protease.

In another embodiment, the prodrug and inhibitor compounds of the invention can be used for the manufacture of a medicament useful for treating diseases such as cancer.

Targeting Cell-Impermeable Prodrug Activation to Tumor Microenvironment Eradicates Multiple Drug-Resistant Neoplasms

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Departments of ¹Immunology and ²Chemistry, The Scripps Research Institute, La Jolla, California; and ³California Peptide Research, Inc., Napa, California

Abstract

The tumor microenvironment is notably enriched with a broad spectrum of proteases. The proteolytic specificities of peptide substrates provide modular chemical tools for the rational design of cell-impermeable prodrugs that are specifically activated by proteases extracellularly in the tumor microenvironment. Targeting cell-impermeable prodrug activation to tumor microenvironment will significantly reduce drug toxicity to normal tissues. The activated prodrug attacks both tumor and stroma cells through a “bystander effect” without selectively deleting target-producing cells, therefore further minimizing resistance and toxicity. Here, we showed that legumain, the only asparaginyl endopeptidase of the mammalian genome, is highly expressed by neoplastic, stromal, and endothelial cells in solid tumors. Legumain is present extracellularly in the tumor microenvironment, associated with matrix as well as cell surfaces and functional locally in the reduced pH of the tumor microenvironment. A novel legumain-activated, cell-impermeable doxorubicin prodrug LEG-3 was designed to be activated exclusively in the tumor microenvironment. Upon administration, there is a profound increase of the end-product doxorubicin in nuclei of cells in tumors but little in other tissues. This tumor microenvironment-activated prodrug completely arrested growth of a variety of neoplasms, including multidrug-resistant tumor *in vivo* and significantly extended survival without evidence of myelosuppression or cardiac toxicity. The tumor microenvironment-activated prodrug design can be extended to other proteases and chemotherapeutic compounds and provides new potentials for the rational development of more effective functionally targeted cancer therapeutics. (Cancer Res 2006; 66(2): 970-80)

Introduction

The local tumor microenvironment differs greatly from that of other tissues. It is enriched in proteolytic activity, is acidic, and is hypoxic. It represents a favorable environment for protease-targeted conversion of inactive compounds to potent cytotoxic agents (1). Targeted activation of prodrug in the tumor extracellular microenvironment can reduce the pressure for selection of

tumor cells lacking expression of the target enzyme, and thereby enhance safety and efficacy of tumor destruction. This goal may be achieved by incorporation of cell membrane permeability inhibiting groups that are subject to catalytic removal by a targeted enzyme present and active in the tumor extracellular microenvironment. Such cell-impermeable targeting prodrugs promise to profoundly reduce active drug access to normal tissues, enhance availability to tumors, and therefore significantly reduce toxicity to normal cells and enhance destruction of neoplastic cells.

Legumain is an entirely novel evolutionary offshoot of the C13 family of cysteine proteases (2). It is well conserved from plants to mammals, including humans. First identified in plants as a processing enzyme of storage proteins during seed germination (3, 4), it was subsequently identified in parasites and then in mammals (5). Legumain is a robust acidic cysteine endopeptidase with remarkably restricted specificity absolutely requiring an asparagine at the P1 site of substrate sequence (5). We found legumain to be highly expressed in a majority of tumors, including carcinomas of the breast, colon, and prostate, and in several central nervous system neoplasms (6); on the other hand, expression is not apparent in normal tissues from which the tumors originated. Legumain is present intracellularly in endosome/lysosome systems and is associated with intracellular protein degradation. Importantly, we showed that legumain is also present extracellularly in the tumor microenvironment, associated with matrix as well as cell surfaces and functional locally in the reduced pH of the tumor microenvironment. Although evident in tumors, this endopeptidase is not detectable in the same tumor cell lines in culture that are used to generate the *in vivo* tumors, inferring an induction of legumain gene expression by the tumor microenvironment (7, 8). In addition to neoplastic cells, we found that legumain is expressed by tumor angiogenic endothelial cells and also here show presence in and on tumor-associated macrophages (9–11), thus presenting multiple local intratumoral cellular targets for prodrug activation (12).

In view of these attractive properties, we designed several legumain-activated prodrugs by covalently linking a cell-impermeable succinyl blocked substrate peptide to the aminoglycoside of doxorubicin. This prototype cell-impermeable targeting tumor microenvironment-activated prodrug is inactive and nontoxic until activated extracellularly by legumain in the acidic tumor microenvironment. Based on *in vivo* activity, LEG-3 (*N*-succinyl- β -alanine-L-alanyl-L-asparaginyl-L-leucyl-doxorubicin) possessed profoundly reduced toxicity and markedly enhanced efficacy compared with doxorubicin in both murine syngeneic CT26 colon cancer and C1300 neuroblastoma models as well as in the human fibrosarcoma HT1080 and doxorubicin-resistant prostate cancer MDA-PCa-2b xenograft models. Mechanistic and pharmacokinetic evidence support the tumor microenvironment-activated prodrug strategy.

Note: W. Wu and Y. Luo contributed equally to this work.

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The potent *in vivo* antitumor efficacy and the improved therapeutic index suggest that LEG-3 represents a promising candidate for highly selective chemotherapeutic eradication of tumors.

Materials and Methods

Reagents and cell lines. Rabbit polyclonal antisera against human legumain as well as 293 cells stably expressing human legumain were kindly provided by Dr. D. Roodman (Department of Medicine and Hematology, University of Texas Health Science Center, San Antonio, TX). A legumain substrate peptide was purchased from Bachem, Inc. (King of Prussia, PA). Doxorubicin was purchased from Sigma (St. Louis, MO) and DMEM was from Invitrogen (Carlsbad, CA). The CT26 murine colon carcinoma, C1300 mouse neuroblastoma cell lines, and the human HT1080 fibrosarcoma cells were purchased from American Type Culture Collection (Manassas, VA).

Antibody preparation. Antilegumain antibodies were prepared by immunizing rabbits with keyhole limpet hemocyanin-conjugated peptide CGMKRASSPVPLPP. A cysteine is added to the legumain sequence. The antilegumain antibodies were affinity purified from resultant antisera using peptide antigen coupled to Ultralink Iodoacetyl Gel from Pierce (Rockford, IL). The bound antibodies were eluted by glycine buffer (100 nmol/L, pH 2.7) and neutralized immediately by adding one-tenth volume of 1 mol/L Tris (pH 7.5).

Western blot. Proteins were dissolved in 2× SDS sample buffer for SDS-PAGE analysis using gradient Tris-glycine gels (8-16%). After electrophoresis, the proteins were transferred to nitrocellulose membranes and blocked with nonfat milk. The antilegumain antiserum was used as the first antibody and was incubated with the membranes for 1 hour (1:1,000 dilution). The blot was washed thrice with PBS, incubated with streptavidin-peroxidase for 15 minutes, and developed by the enhanced chemiluminescence method (Sigma).

Flow cytometry analysis. Single-cell suspensions were prepared from organs and tumor tissues as previously reported (6). Rabbit antilegumain antisera diluted 1:5,000 or antigen purified antilegumain antibody at 0.5 µg/mL in PBS are used to detect legumain. This is followed by FITC-conjugated goat anti-rabbit IgG diluted 1:5,000 in PBS (BD Pharmingen, La Jolla, CA). For CD14 staining, the phycoerythrin-conjugated anti-mouse CD14 antibody diluted 1:3,000 in PBS was used (BD Pharmingen).

Immunohistochemical analysis. Immunohistochemical staining was done on 5-µm-thick frozen sections on poly-L-lysine slides. For endothelial identification, biotinylated rat anti-mouse CD31 monoclonal antibody (MEC 13.3) was used with Texas red-conjugated streptavidin as the secondary reporting reagent. For staining of legumain, rabbit polyclonal anti-legumain antisera was used at 1:500 dilution or antigen-purified antilegumain polyclonal antibody at 0.5 µg/mL and visualized with FITC-conjugated goat anti-rabbit antibody. For the identification of tumor-associated macrophage, rat anti-mouse CD68 antibody was used and followed by an antirat antibody conjugated with Texas red. For identification of collagen I, a biotinylated rabbit anti-mouse collagen I antibody was used at 1:250 dilution and visualized with Texas red-conjugated streptavidin. The slides were analyzed by laser scanning confocal microscope (Bio-Rad, Hercules, CA).

Terminal deoxynucleotidyl transferase-mediated nick end labeling analysis. DNA fragmentation caused by apoptosis was detected by terminal deoxynucleotidyl transferase-based, *in situ* cell death detection kit (Roche Applied Science, Mannheim, Germany). The procedure was done according to the instructions of the manufacturer. Briefly, the sections were treated with protein K solution (10 µg/mL in 10 mmol/L Tris/HCl, pH 7.4) for 15 minutes and followed by 15-minute incubation with terminal deoxynucleotidyl transferase (TdT)-mediated nick end labeling (TUNEL) reaction mixture containing TdT and FITC-dUTP. The TUNEL alkaline phosphatase kit (Roche Applied Science) was used for the conversion of fluorescence-based TUNEL detection into a colorimetric labeling. The conversion was achieved by binding of an antifluorescein antibody to FITC-dUTP. The antibody is labeled with alkaline phosphatase. The signals were visualized with Fast Red (Vector Laboratories, Burlingame, CA).

Prodrug synthesis. The synthesis of the succinyl version of the prodrug used the azide method to protect the peptide from racemization. In

principle, the N-protected amino acids or peptide esters are converted by hydrazine derivatization to an acid hydrazide. Subsequent reaction with HNO₂ or derivatives leads to anacylazide. Thus, the succinyl-Ala-Ala-Asn-Leu-N₂H₃ peptide was prepared by using liquid phase synthesis. It was directly used to synthesize the target compound. An example of the synthesis is as follows. Solution A: 1,040 mg succinyl-Ala-Ala-Asn-Leu-N₂H₂F was dissolved in a small amount of dimethylformamide (DMF) cooled to -10°C and 1.5 mL of 4 N HCl dioxane was added followed by 2.1 mmol/L isoamyl nitrite. The mixture was stirred for 30 to 40 minutes at -10°C and then the pH was carefully adjusted to 7.5 with diisopropyl ethylamine. Solution B: 1,210 mg doxorubicin acetate was dissolved in a small amount of DMF at room temperature, the pH was adjusted to 7.5 with DIPEA, and the solution was chilled to -10°C. Solutions A and B were combined and the pH was readjusted to 7.5 and monitored throughout the reaction. The reaction mixture was allowed to warm to 4°C and allowed to stand overnight. High-performance liquid chromatography (HPLC) analysis indicated ~80% completion of the reaction within 24 to 48 hours. The reaction mixture was then diluted 10-fold with 0.1% trifluoroacetic acid (in H₂O) and applied directly onto preparative HPLC. A linear acetonitrile gradient was used to elute the target compound. Fractions were analyzed for purity, combined, and lyophilized. HPLC, amino acid analysis, and mass spectrometry were done on the lyophilized powder.

Cytotoxicity assays. The WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolol-1,3-benzene disfonate] cell proliferation reagent (Roche Molecular Chemicals, Mannheim, Germany) was used to determine cell proliferation by quantization of cellular metabolic activity. Control 293 cells and legumain⁺ 293 cells were cultivated in microtiter plates (5 × 10³ per well in 100 µL) and were incubated with serial concentrations of LEG or doxorubicin for 48 hours. Subsequently, 10 µL WST-1 solution (1 mg/mL WST-1, 25 µmol/L methylidibenzopyrazine methyl sulfate) was added per well and mixtures were incubated for an additional 4 hours. The tetrazolium salt WST-1 was cleaved by the mitochondrial succinate-tetrazolium-reductase system to formazan in cells in direct correlation with the number of metabolically viable cells in the culture. The amount of formazan salt was quantified in three replicates by absorbance at 450 nm using a microplate reader (Molecular Devices, Palo Alto, CA). All results were derived from replicate experiments with similar results.

Doxorubicin and LEG-3 uptake assay. The legumain⁺ 293 cells or control 293 cells (2.5 × 10⁵ per well) were seeded in six-well plates. The culture plates were then incubated for 24 hours at 37°C and 5% CO₂ and the medium in each well was replaced with 2 mL of serum-free, antibiotic-free medium containing various concentrations of doxorubicin or LEG-type compounds. The cells were incubated 1.5 hours then washed thrice with 2 mL cold PBS. At this point, cell nuclei doxorubicin positivity can be analyzed by fluorescent microscopy. For quantitative assays, the cells were then lysed by adding 0.5 of water and gently rotated on an orbital shaker for 5 minutes at room temperature. The lysed cells were added to 1.5 mL acidified ethanol and incubated at 4°C in the dark for 3 hours. Total doxorubicin and LEG content was measured fluorometrically using a Perkin-Elmer LS-50-B spectrofluorometer (excitation: 470 nm; emission: 590 nm). Fluorescence intensity was translated to drug concentration by use of a standard curve prepared from doxorubicin and LEG solutions in cell lysates that were not previously exposed to the drug. Results are expressed as the mean ± SD of at least three replicates for each experiment.

Determination of marrow toxicity. Groups of healthy BALB/c mice (*n* = 4) were injected i.p. with a single dose of LEG-3 (49.4 or 4.94 µmol/kg) or doxorubicin (3.4 µmol/kg). On day 7, retro-orbital sinus blood samples were collected into 10 mmol/L EDTA and were counted by hemocytometer after lysis of RBCs with an acidified methyl violet solution.

Determination of maximum tolerable dose. Four six-week-old BALB/c mice were used for each experimental group. The mice were weighed individually and the average weight of the group is used to determine the exact doses. Mice were given i.p. injection daily for 5 days. The maximum tolerable dose (MTD) is defined as the maximum drug dose administered to non-tumor-bearing mice once daily for 5 consecutive days without mortality.

Tissue distribution. LEG compounds or doxorubicin was injected i.p. into mice; 12 hours later, the animals were perfused and the doxorubicin autofluorescence was measured following homogenization in 50% ethanol and then diluted with an equal volume of 50% ethanol containing 0.6 mol/L HCl. Fluorescence measurements were obtained with excitation at 470 nm and emission at 590 nm; concentrations were derived by conversion from a doxorubicin standard curve. Tissues from saline-injected mice provided controls. Blood samples were to 0.75 mL with PBS, centrifuged, the pellets washed with PBS, and doxorubicin was extracted with ethanol and 0.3 mol/L HCl.

Animal models. The CT26 syngeneic murine colon carcinoma model was generated and maintained in The Scripps Research Institute animal facility. This model was produced in BALB/c mice ages 4 to 6 weeks injected with 5×10^5 CT26 tumor cells per s.c. site on the back. The C1300 mouse neuroblastoma model was generated in A/J mice by s.c. injection of 5×10^5 C1300 cells per site on the back. Treatment was initiated when the tumors reached 4 mm in diameter through bolus i.p. (syngeneic tumors) or i.v. (human tumors) injections of the indicated reagents. Treatment was thrice per week for 2 weeks. The human HT1080 fibrosarcoma was xenografted in BALB/c *nu/nu* mice obtained from The Scripps Research Institute breeding colony. HT1080 cells, 1×10^6 per site, were inoculated s.c. on the back. The MDA-PCa-2b human prostate carcinoma model was generated in WEHI nude mice and these cells (10^6) were also injected s.c. Tumor growth and signs of physical discomfort were monitored daily including for any gross evidence of tumor necrosis, local tumor ulceration, as well as evidence of toxicity including the mobility of animals, response to stimulus, piloerection, eating, and weight. These procedures have been reviewed and approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute. All the experiments were conducted in The Scripps Research Institute facilities, which are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. The Scripps Research Institute maintains an assurance with the Public Health Service and is registered with the Department of Agriculture and is in compliance with all regulations relating to animal care and welfare.

Statistical analysis. Statistical significance of data was determined by the two-tailed Student's *t* test, except for statistical significance of survival curves, which used the log-rank test using GraphPad Prism version 3.00 (GraphPad Software, San Diego, CA).

Results

Legumain is highly expressed by cells in the tumor microenvironment. We have reported that legumain is expressed *in vivo* by tumor cells and proliferating endothelial cells intracellularly as well as on their cell surfaces (6). In contrast, legumain is not detectable in or on CT26, C1300, HT1080, and MDA-PCa-2b as well as eight other tumor cell lines in culture, the same cells used to generate a set of legumain-expressing tumors *in vivo* (Fig. 1A). Further, this endopeptidase is not detectable in large-scale survey panels of tumor cell lines in culture, except for THP-1 cells, based on a search of the National Cancer Institute tumor cell expression database.

Using immunohistochemical analysis, legumain is usually present in tissue sections of human colon carcinomas, neuroblastomas, fibrosarcomas, and prostate carcinomas, representative of the types of neoplasms investigated in the present study. Legumain expression was not detectable in the normal tissues of origin for these neoplasms (6). The remarkable local expression of legumain not only by neoplastic cells but also by stromal cells in association with tumor development *in vivo* infers that this response is in response to novel local aspects of the tumor microenvironment. Legumain is found on the CT26 tumor cell surfaces where it is effectively removed by 30 minutes collagenase digest at 57°C (Fig. 1B). This further establishes the cell surface association of legumain in contrast to the previously identified intracellular

localization of legumain, as there is no transmembrane domain or prior evidence of secretion or plasmalemma localization of this lysosomal protease.

To analyze the extracellular localization of legumain in tumors and normal organs, flow cytometry was used to analyze cell surface legumain in single-cell suspensions prepared from tumors, bone marrow, spleen, and kidney, as well as cultured tumor cells. Despite demonstrable intracellular legumain by renal tubular epithelial cells, <2% of isolated viable cells were very weakly positive for cell surface legumain. Spleen cells have considerably less legumain than renal cells; however, ~1% to 2% of spleen cells are weakly positive for cell surface legumain. Furthermore, cell surface legumain is not detectable on cells derived from bone marrow nor is it found on cultured CT26 cells. In contrast, 40% of intact viable cells derived from *in vivo* CT26 tumors were strongly positive for cell surface legumain (Fig. 1C). A similar pattern was observed for all tumors examined (data not shown), indicating that cell surface and extracellular legumain is uniquely abundant only in tumors. Using confocal microscopy analysis, we have described previously that legumain is expressed by tumor vascular endothelial cells (Fig. 2A;

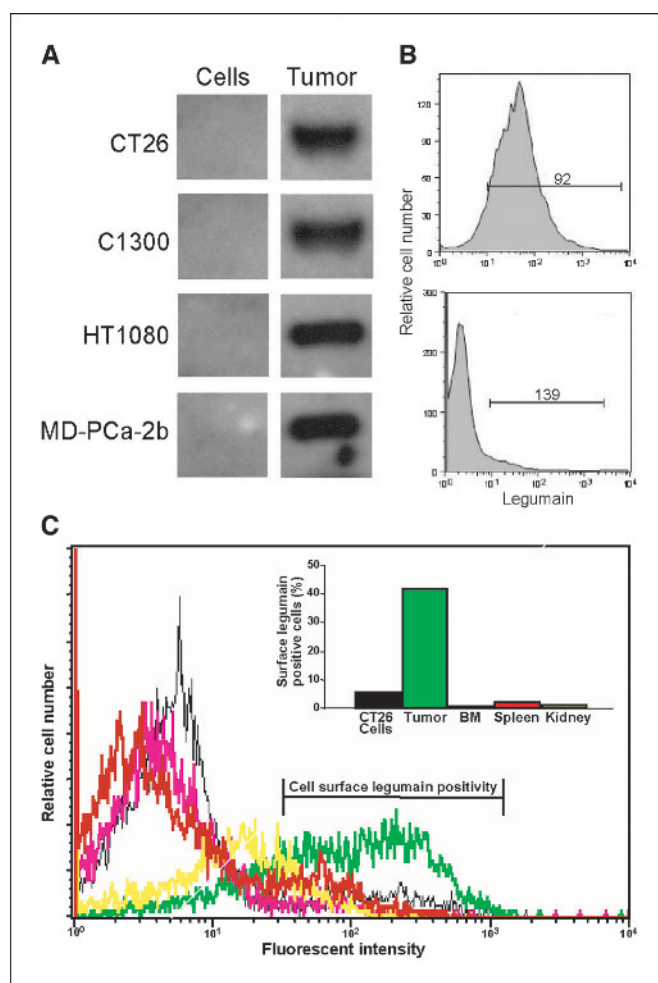


Figure 1. Legumain expression and cell surface association. **A**, Western blot analysis of cultured tumor cells and corresponding *in vivo* tumor-derived cells. **B**, flow cytometry analysis for cell surface legumain of single-cell suspensions from CT26 tumors with (bottom) and without (top) collagenase treatment. **C**, flow cytometry analysis of single-cell suspensions prepared from tumor, bone marrow (BM), spleen, and kidney as well as cultured CT26 tumor cells for cell surface legumain. Columns (inset), percentage of cells positive for surface legumain.

ref. 6). Here, we showed that legumain is expressed by tumor-associated macrophages in tissue sections from CT26 tumors by dual staining with antilegumain and anti-CD68 antibodies (Fig. 2B). Secreted legumain is present in the tumor stroma associating with extracellular matrix proteins, such as collagen I (Fig. 2C). Legumain expression is absent in normal peripheral blood monocytes. Using flow cytometry, legumain is found on the surface of viable endothelial cells and tumor-associated macrophages using both antilegumain antibody and anti-CD31 antibody or anti-CD14 antibody, respectively (Fig. 2D). Interestingly, legumain on endothelial cell and tumor-associated macrophage surfaces is resistant to removal by collagenase, suggesting a mode of cell surface association (Fig. 2E) distinct from that of tumor cells where legumain is removed by collagenase.

LEG-3 is activated by tumoral legumain. The ability of the cell-impermeable targeting tumor microenvironment prodrug LEG-3 (Fig. 3A) to kill tumor cells was evaluated first in cell culture (Fig. 3B). With both legumain transfected and control nonproducing cells, we determined the median effective concentration representing the amount of LEG-3 or doxorubicin required for 50% cell death (EC_{50} ; Table 1). LEG-3 was virtually noncytotoxic to control cells not expressing legumain. However, for cells that were transfected with legumain cDNA and express cell surface legumain, cytotoxicity was significant and EC_{50} levels were close to those observed for doxorubicin, indicating an efficient conversion of the LEG-3 prodrug. The requirements for activation were shown using an alternate peptide sequence not hydrolyzed by legumain [LEG-4 (*N*-succinyl- β -alanyl-L-asparaginyl-L-leucyl-doxorubicin);

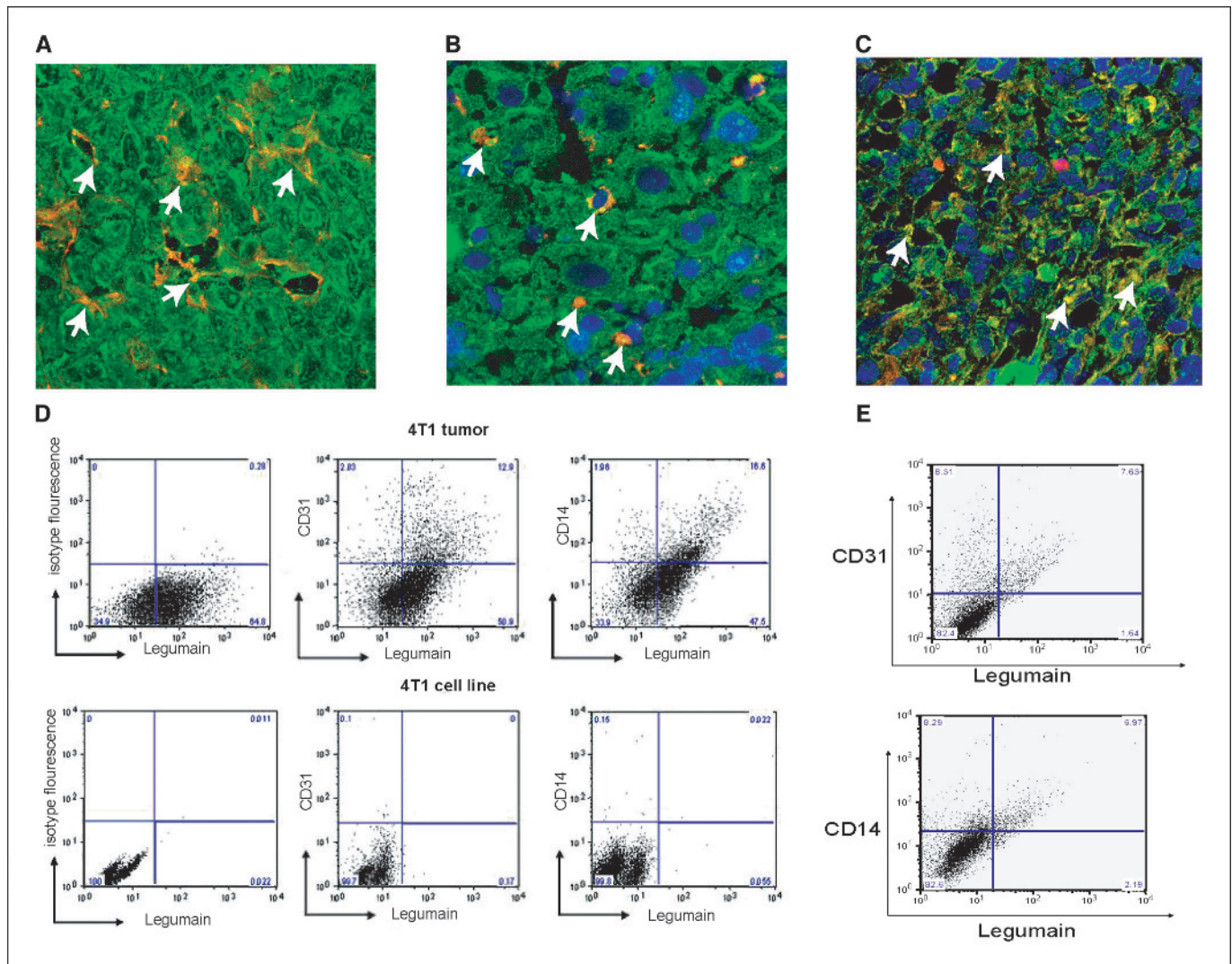


Figure 2. Legumain is expressed by stromal cells in tumors. *A*, double staining of antilegumain antibody (green) and anti-CD31 antibody (red) identifies endothelial cells. Arrows, tumor vascular endothelial cells expressing legumain in tumor ($\times 400$). *B*, double staining of antilegumain antibody (green) and anti-CD68 antibody (red) identifies legumain-expressing, tumor-associated macrophages (arrows; $\times 600$). Arrows, tumor-associated macrophage cells expressing legumain. *C*, double staining of antilegumain (green) with anticollagen I antibody (red; $\times 400$). Colocalization of legumain with collagen I in the extracellular matrix (yellow). *D*, two-dimensional analysis of single-cell suspensions prepared from 4T1 *in vivo* mouse mammary tumor following mechanical dissociation and of 4T1 tumor cells from tissue culture. The presence of cell surface legumain in tumor vascular endothelial cells and tumor-associated macrophages is shown with the presence of cells that are both legumain⁺ and CD31⁺ as well as cells that are both legumain⁺ and CD14⁺, respectively. *E*, two-dimensional flow cytometry of single-cell suspensions prepared from 4T1 tumors using collagenase digestion. Legumain-associated endothelial cells are represented by the group of cells that are legumain⁺ and CD31⁺. Legumain-associated, tumor-associated macrophages are represented by legumain⁺ and CD14⁺ cells.

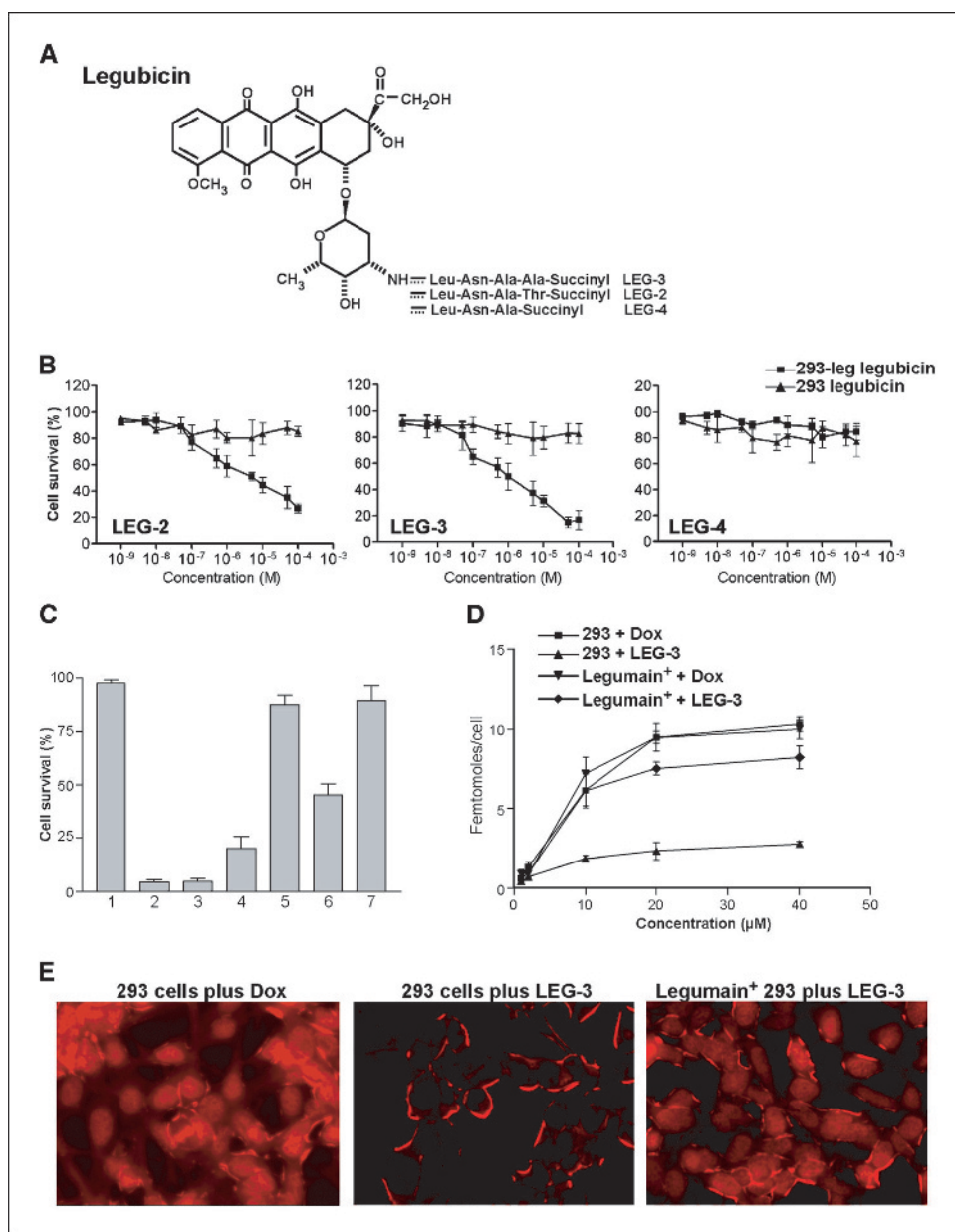


Figure 3. LEG family compounds. **A**, schematic structure of these LEGs. LEG-2 and LEG-3 are oligopeptidic derivatives of doxorubicin that are cell impermeable and can be hydrolyzed to leucine-doxorubicin by extracellular legumain. LEG-4 is similar but is not subject to legumain hydrolysis. **B**, cytotoxicity of LEG compounds using legumain⁺ 293 cells and control wild-type 293 cells. **C**, legumain-mediated LEG activation and inhibition by cystatin. **D**, cell uptake assay of LEG-3 compared with doxorubicin (Dox). **E**, localization of doxorubicin in cell nuclei is visualized by autofluorescence (red). In contrast, LEG-3 is not internalized by legumain-negative cells exposed to LEG-3 and they lack nuclear positivity for end product doxorubicin despite presence of extracellular fluorescent signal of the doxorubicin present in the LEG-3.

Fig. 3B] and also by inhibition of LEG-3 activation following inhibition of legumain function (Fig. 3C).

LEG-3 is activated extracellularly. Tumor cell uptake of LEG-3 was compared with doxorubicin. When added to cell cultures, doxorubicin rapidly entered cells. In contrast, LEG-3 remained extracellular, consistent with its observed lack of cytotoxicity. In contrast, when LEG-3 was added to legumain-expressing cells in culture, the end product doxorubicin was found in cells (Fig. 3D and E).

Legumain is selective and functional in the tumor microenvironment. When doxorubicin was administered as a single i.v. bolus, the plasma concentration very rapidly declined followed by a low concentration and slowly cleared phase, consistent with observations by other investigators (13, 14). The initial decline of infused LEG-3 was much slower, which is attributed to reduced tissue uptake (Fig. 4A). The content of LEG-3 in tumor tissues was determined 12 hours postinjection in mice bearing CT26 tumors. There was significant doxorubicin content in tumors, in contrast to

many tissues, including heart, kidney, liver, and brain. The MTD of doxorubicin and a molar equivalent amount of LEG-3 were given i.v. For LEG-3, the amount of drug present in tumors was >10-fold greater than that for doxorubicin administration. LEG-3 was greatly reduced in cardiac tissue (Fig. 4B). Because of the reduced normal tissue uptake and toxicity, larger quantities of LEG-3 could be administered, which resulted in higher drug content in cells within tumors compared with that achieved for doxorubicin administration. Drug accumulation in tissues and tumor was visualized by doxorubicin autofluorescence (Fig. 4C). The data indicate that legumain is selectively found in the tumor microenvironment, and LEG-3 is processed to its cell-permeable Leu-doxorubicin derivative based on the presence of cytoplasmic doxorubicin, which, following processing to the end product doxorubicin, translocated to the nucleus based on intranuclear fluorescence.

In vivo toxicity of LEG-3 and doxorubicin. LEG-3 is significantly less toxic than doxorubicin when evaluated *in vivo*.

Table 1. *In vitro* and *in vivo* toxicity of LEG-3 compared with doxorubicin**A.** Estimated IC₅₀ (μmol/L) of doxorubicin and LEG-3

| | 293 cells | Legumain+293 cells | HTP cells |
|------------------|-----------|--------------------|-----------|
| Dox | 1.2 | 1.4 | 1.5 |
| LEG-3 | >100 | 3.2 | 9.6 |
| LEG-3 + cystatin | >100 | 64.4 | 59.2 |
| LEG-4 | >100 | >100 | >100 |

B. Estimated MTD and LD₅₀ of Dox and LEG-3 (μmol/kg) in BALB/c male mice

| | Dox | | LEG-3 | |
|---------|------|------------------|--------|------------------|
| | MTD | LD ₅₀ | MTD | LD ₅₀ |
| I.p. | 9.8 | 17.2 | >197.4 | >197.4 |
| I.p. 5× | 2.8 | 3.4 | 98.7 | >197.4 |
| I.v. | 18.2 | 25.8 | >197.4 | >197.4 |
| I.v. 5× | 3.2 | 5.1 | 74.0 | 197.4 |

C. Comparison of gross toxicity of mice treated with Dox and LEG-3

| | | | | | |
|-------------------------|------|-----|------|------|-------|
| Controls | | | | | |
| Average weight loss (%) | 4 | | | | |
| Dox | | | | | |
| Dose (μmol/kg) | 1.72 | 5.4 | | | |
| Average weight loss (%) | 24 | 28 | | | |
| Death (%) | 35 | 75 | | | |
| LEG-3 | | | | | |
| Dose (μmol/kg) | 1.97 | 4.9 | 49.4 | 98.7 | 197.4 |
| Average weight loss (%) | 3 | 5 | 7 | 8 | 16 |
| Death (%) | 0 | 0 | 0 | 10 | 50 |

Abbreviation: Dox, doxorubicin.

When given by six repeat i.v. administrations, LEG-3 had a much higher cumulative MTD and reduced LD₅₀ compared with doxorubicin (Table 1). Bone marrow and cardiac toxicity of LEG-3 was examined and compared with doxorubicin. Also, the cytotoxic effect of LEG-3 on peripheral blood leukocyte counts (WBC) was assessed and compared with that of doxorubicin. There were no reductions of total WBC counts in mice treated six times in 12 days with 4.94 μmol/kg LEG-3, and only a slight reduction observed in mice treated with a 10-fold higher dose. In contrast, there was >50% reduction of WBC counts in the group of mice receiving as little as 3.4 μmol/kg doxorubicin by the same schedule (Fig. 5A). LEG-3 produced little evidence of myelosuppression compared with its parent compound doxorubicin. In cardiac tissue, mice similarly treated with 49.4 μmol/kg of LEG-3 exhibited no histologic evidence of cardiac toxicity in contrast to profound cardiac myocyte vacuolization and cell death in mice treated with doxorubicin at 3.4 μmol/kg (Fig. 5B). Cardiac myocytes of mice treated with doxorubicin showed marked apoptosis, which was not observed for LEG-3-treated mice (Fig. 5B).

***In vivo* efficacy is dependent on legumain-specific activity.** To further characterize the requirements for legumain activation of LEG-3 *in vivo*, two LEG-type compounds differing in composition of the peptidyl element of the compound were evaluated. LEG-2 (*N*-succinyl-β-alanyl-L-threoinyl-L-Asparaginyl-L-Leucyl-

doxorubicin) and LEG-4 were analyzed for *in vivo* efficacy in tumor-bearing mice. The LEG-4 compound is not cleaved by legumain and was devoid of tumoricidal activity (Fig. 6A), whereas LEG-2 and LEG-3, which are converted by legumain, showed *in vivo* tumoricidal efficacy (Fig. 6B and C).

LEG-3 has effective tumoricidal activity against diverse tumors *in vivo*. The potential therapeutic efficacy of LEG-3 was evaluated in both syngeneic rodent tumor models and human tumor xenograft models. At a well-tolerated dose (49.4 μmol/L/kg) administered six times in 12 days, LEG-3 effectively arrested growth of murine CT26 colon carcinoma in BALB/c mice and produced complete tumor eradication in some (Fig. 6C). A similar level of efficacy was also observed for the murine C1300 neuroblastoma in A/J mice (Fig. 6D) where LEG-3 treatment induced massive tumor cell death (Fig. 6C and D), tumor eradication, and marked enhancement of survival (Fig. 6E and F).

The *in vivo* efficacy of LEG-3 on human tumor xenografts in athymic *nu/nu* mice was assessed and compared with doxorubicin. Legumain protein was not detectable in either HT1080 or MDA-PCa-2b cells in culture. However, robust legumain expression was observed by immunohistochemistry for *in vivo* tumors propagated from these cells. Indeed, LEG-3 produced potent tumoricidal activity against the HT1080 fibrosarcoma, a fast-growing tumor and a model that is traditionally sensitive to doxorubicin therapy

(Fig. 7A). On the other hand, human prostate carcinomas are frequently resistant to doxorubicin therapy. MDA-PCa-2b prostate carcinoma, a known doxorubicin-resistant tumor (15), failed to respond to doxorubicin *in vivo*. However, administration of LEG-3 led to complete growth arrest (Fig. 7B). LEG-3 was effective and frequently resulted in complete tumor eradication with marked enhancement of survival of the HT1080 as well as the MDA-PCa-2b tumor-bearing mice (Fig. 7C and D). Toxicity of LEG-3 based on weight loss and mortality was negligible (Fig. 7E).

Discussion

This cell-impermeable targeting tumor microenvironment-activated prodrug strategy depends on targeting as well as specific catalytic function at a pH <6.8 of a quite unique endopeptidase that is selectively expressed extracellularly in the tumor microenvironment. There, it can convert an effectively designed cell-impermeable prodrug (LEG-3) to a cell-permeable prodrug (Leu-doxorubicin), which, in turn, is converted to the active tumoricidal end product doxorubicin and translocated to cell nuclei for induction of cell death. There are distinct advantages to this strategy in that tissue uptake can occur only in the tumor or comparable pathologic microenvironment. To achieve continuous growth and remodeling, the tumor microenvironment is enriched with a variety of proteases. There are nearly 500 proteases identified thus far in the human genome (16, 17), and many have been associated with the local tumor microenvironment and seem important for tumor invasion and metastasis (18, 19). Drug access to solid tumors is relatively efficient, limited only by diffusion

barriers. Compounds, such as LEG-3, converted and activated in the tumor microenvironment can access and be converted by both neoplastic and associated endothelial and stromal cells to produce substantial bystander effects. Whereas most cytotoxic drugs are designed to be cell permeable, here we diminished cell permeability, increased hydrophilic properties, and increased drug solubility to minimize tissue uptake of LEG-3. This correlates with the slower clearance from the blood as well as greatly diminished tissue accumulation.

The design of an effective tumor microenvironment-activated prodrug of this type requires knowledge of the selectivity and expression of the enzyme target, including not only functional characteristics but also *in vivo* distribution under physiologic and pathologic conditions. In respect to these issues, extracellular accessible legumain represents a promising candidate target because it is the only asparaginyl endopeptidase in the mammalian genome. We discovered that legumain is highly expressed in the majority of solid tumors (6). It is a robust acidic cysteine protease, one that is overexpressed by neoplastic cells as well as intratumoral endothelial cells and macrophages. It requires for function the local acidic tumor microenvironment and is found associated with both the extracellular matrix and cell surfaces in the tumors. In normal tissues, such as kidney, legumain is present in proximal tubular epithelial cells but only as an intracellular lysosomal protein. Although not found, extracellular legumain in normal tissues would be functionally inactive at physiologic pH (5) and such protein that may escape the tumor microenvironment would be inactive for this same reason. Legumain activity in normal tissues could be inhibited by cysteine protease inhibitors, such as

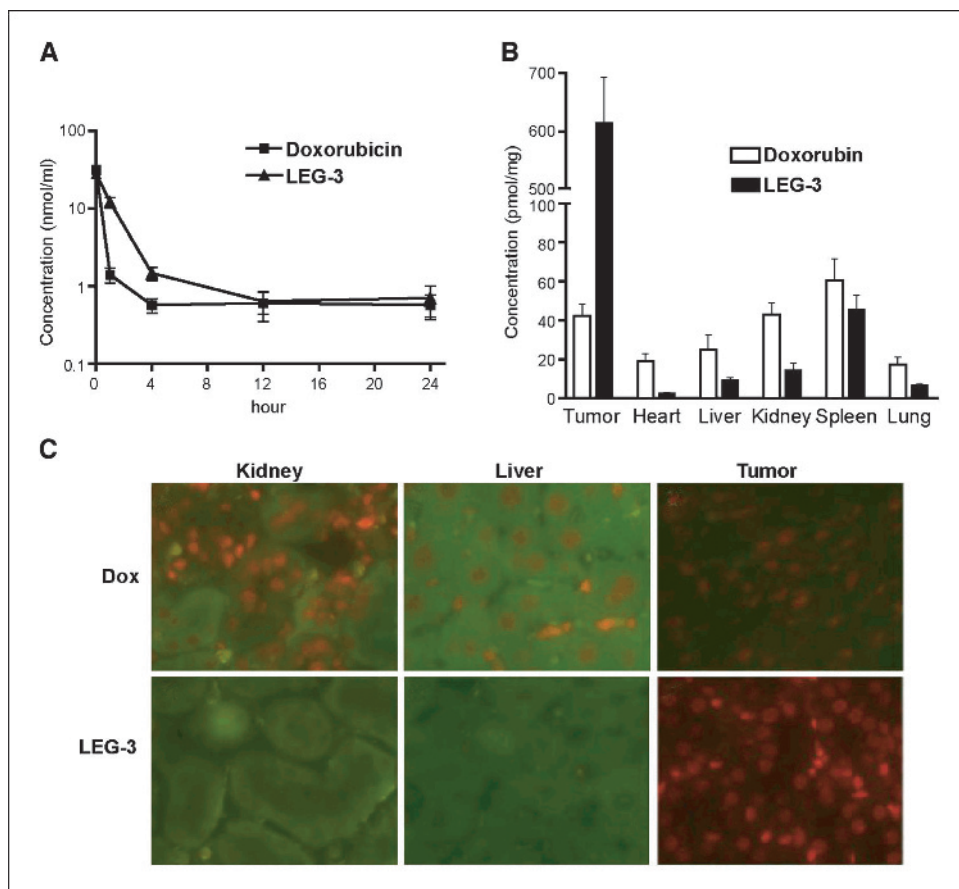
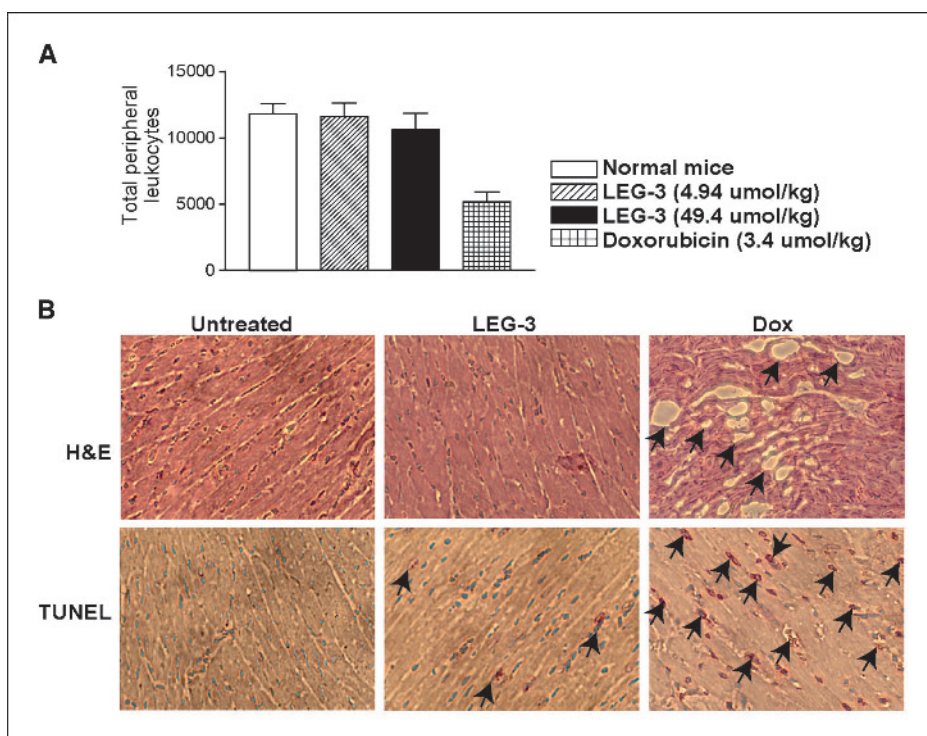


Figure 4. *In vivo* distribution and pharmacokinetics of LEG-3. **A**, plasma pharmacokinetics of LEG-3 compared with doxorubicin. **B**, accumulation of LEG-3 and doxorubicin in organs and tumors of mice bearing CT26 colon carcinomas. **C**, presence of doxorubicin and LEG-3 are visualized with autofluorescence of doxorubicin (red).

Figure 5. LEG-3 lacks *in vivo* toxicity of doxorubicin. **A**, myelosuppression of LEG-3 and doxorubicin in mice were assessed by determination of peripheral blood leukocyte counts. **B**, cardiac toxicity was shown by the presence of vacuolar degeneration of myocytes in H&E-stained sections resulting from chronic doxorubicin treatment, which was notably absent in LEG-3-treated mice. TUNEL analysis of cardiac tissue from mice treated with doxorubicin also showed marked apoptosis of myocytes (arrows, red apoptotic nuclei). This was very infrequent in LEG-3-treated mice.



cystatin C (20), whereas such inhibitors are commonly down-regulated in tumors (21). In addition to legumain expression, the function of reticuloendothelial system can contribute to the relative higher spleen uptake of prodrug and doxorubicin. However, we did not observe gross spleen enlargement or atrophy in prodrug-treated mice; there is no apparent cellular depletion of lymphoid or myelogenous lineages. Therefore, the legumain-activated prodrugs do not seem to have an enhanced toxicity toward spleen. The cell-impermeable targeting tumor microenvironment-activated prodrug strategy need not be restricted to doxorubicin as the cytotoxic end product. Other compounds are compatible with modification to achieve a similar tumor microenvironment-activated prodrug mode of action. Of particular interest for incorporation into this strategy are a number of highly cytotoxic compounds that have been developed based on their *in vitro* activity as exemplified by duocarmycin (22, 23).

Tumor-associated proteases have been recognized increasingly as potential targets for selective activation of prodrugs. Thus far, prodrugs were reported that are activated by prostate-specific antigen (24, 25), cathepsin (26–28), plasmin (29), and by undefined tumor-associated proteases (14, 30, 31). Here, we explored the potential of legumain to serve as a tumor-selective target and evaluated the potential of the tumor microenvironment-activated prodrug strategy. The lead candidate compound LEG-3 was analyzed *in vitro* and *in vivo*. The aminoglycoside at position C-3' is critical for the ability of doxorubicin to integrate into DNA and to interfere with DNA Topo II and to overcome multiple drug resistance (32, 33). The addition of the legumain substrate peptidyl structure to the C-3'-NH₂ abolishes this function. It is critical to our design that charge and the succinyl cap of LEG-3 prevent cell entry. Also, the peptidyl element must not be susceptible to hydrolysis by any other enzyme. In tumor cells overexpressing legumain, LEG-3 was effectively rendered cell permeable, the resultant Leu-doxorubicin prodrug was processed with transloca-

tion of end product doxorubicin to nuclei, thereby mediating cytotoxicity. LEG-3 is effective against a variety of multidrug-resistant solid tumors, including not only rapidly progressing syngeneic rodent tumors but also slower growing human tumor xenografts in immunodeficient mice. The specific activation of LEG-3 by legumain in tumors results in higher drug delivery to tumor cells and resultant cytotoxic destruction.

Our data clearly showed that LEG-3 is a tumor microenvironment-activated prodrug that is selectively catalytically converted to end product doxorubicin in the tumor microenvironment. LEG-3 is not found in any significant amount in normal tissues presumably as a result of its cell impermeability. Based on LD₅₀, the toxicity of LEG-3 in the mouse was reduced >10-fold compared with doxorubicin. LEG-3 also exhibited a slower initial reduction in plasma concentration than doxorubicin consistent with the relative tissue impermeability. For cardiac tissue, LEG-3 accumulation was reduced >15-fold. Cardiomyopathy and the development of congestive heart failure is associated clinically with cumulative doxorubicin dosage in excess of 500 to 550 mg/m², a level readily achieved when required for tumors responsive to the drug, and is the major limitation for therapeutic use of doxorubicin and other anthracyclines (34). This is a notable advantage of compounds like LEG-3 because it is far less cardiotoxic. The toxicity of DNA-intercalating drugs is particularly injurious to tissues with high cell proliferation as exemplified by severe myelosuppression. We found little effect of LEG-3 on cells of myeloid lineage, as mice showed negligible reduction in peripheral blood or marrow myeloid cells at elevated therapeutic doses.

Another advantage of a tumor microenvironment-activated prodrug, such as LEG-3, over doxorubicin is the increased plasma persistence, allowing longer tumor exposure to enhance targeting. Based on the reduced toxicity, larger cumulative dosage of LEG-3 can be administered more rapidly. Consequently, significantly greater tumor inhibition and destruction have been observed for

LEG-3 in syngeneic murine colon carcinoma and neuroblastoma models without demonstrable toxicity. LEG-3 was also highly effective against human fibrosarcoma and a doxorubicin-resistant human prostate carcinoma in xenograft models where high levels of intratumoral legumain are present.

Legumain is also produced by endothelial cells and tumor-associated macrophages in the tumor microenvironment. These cells constitute additional local intratumor targets for competent drug activation and therapeutic effects, including tumor microvascular destruction. Evidence that tumor-associated macrophages can be directly tumoricidal and also stimulate tumoricidal activity

of T cells is questionable. To the contrary, tumor cells frequently are able to evade the activity of tumor-associated macrophages (11). In some tumors, tumor-associated macrophages account for as many as 50% of cells. Further, evidence has emerged for a symbiotic relationship between tumor cells and tumor-associated macrophages. Tumor cells attract tumor-associated macrophages, which, in turn, provide a considerable array of growth factors and cytokines that can facilitate tumor cell survival. Tumor-associated macrophages have been reported to respond to microenvironmental factors in tumors, such as hypoxia, by producing growth factors including vascular endothelial growth factor (35–37), enzymes such

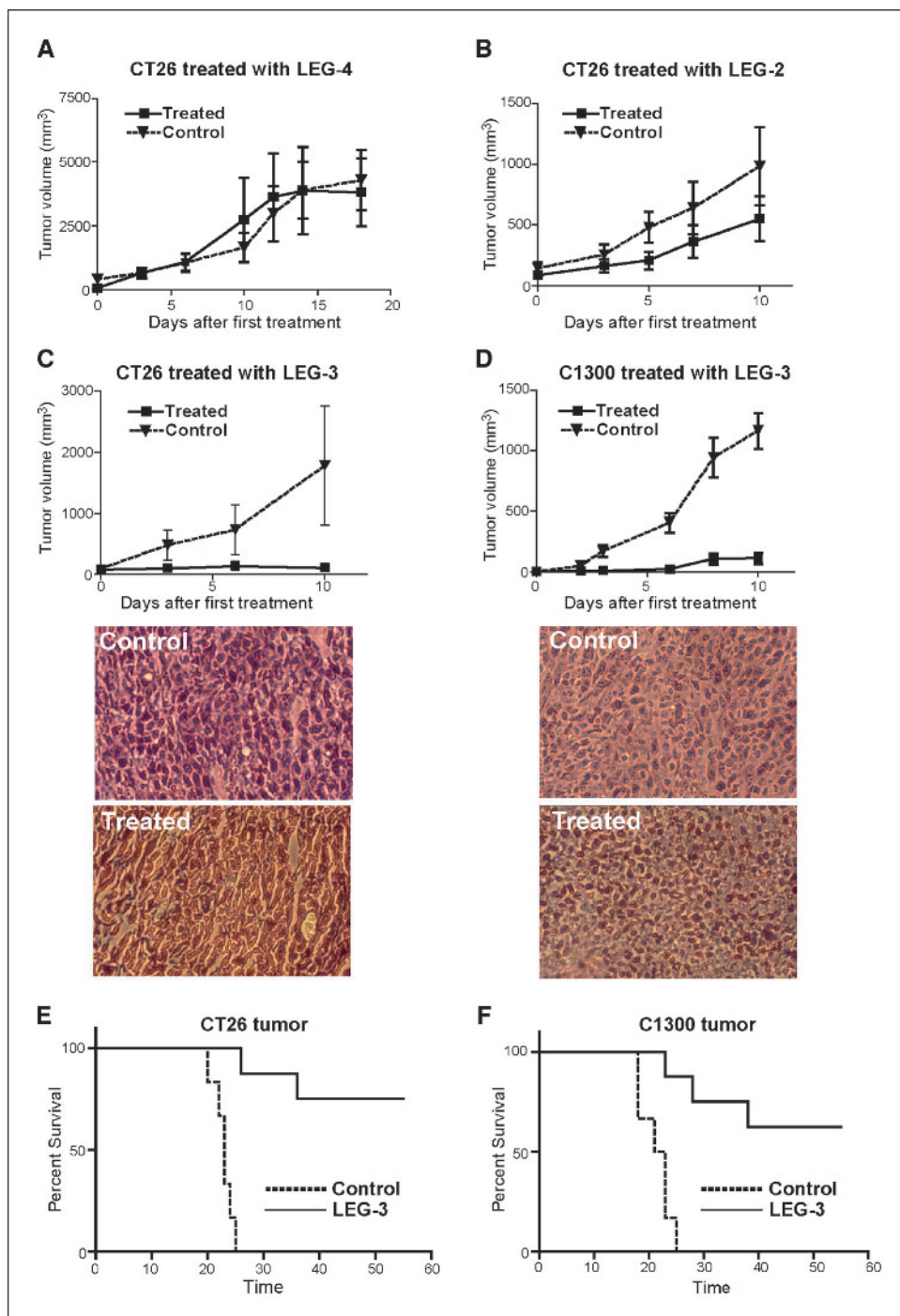


Figure 6. *In vivo* specificity and efficacy of LEG-3 in syngeneic mouse tumor models. *A*, *in vivo* effects of LEG-4 in mice bearing CT26 tumors ($n = 8$). *B*, LEG-2 ($n = 8$). *C*, LEG-3 ($n = 8$). H&E-stained sections of control versus treated tumors. *D*, *in vivo* effect of LEG-3 in A/J mice bearing C1300 neuroblastoma ($n = 8$). H&E-stained sections of control versus treated tumors. *E*, survival analysis of CT26 tumor-bearing mice treated with LEG-3. *F*, survival analysis of C1300-bearing mice treated with LEG-3.

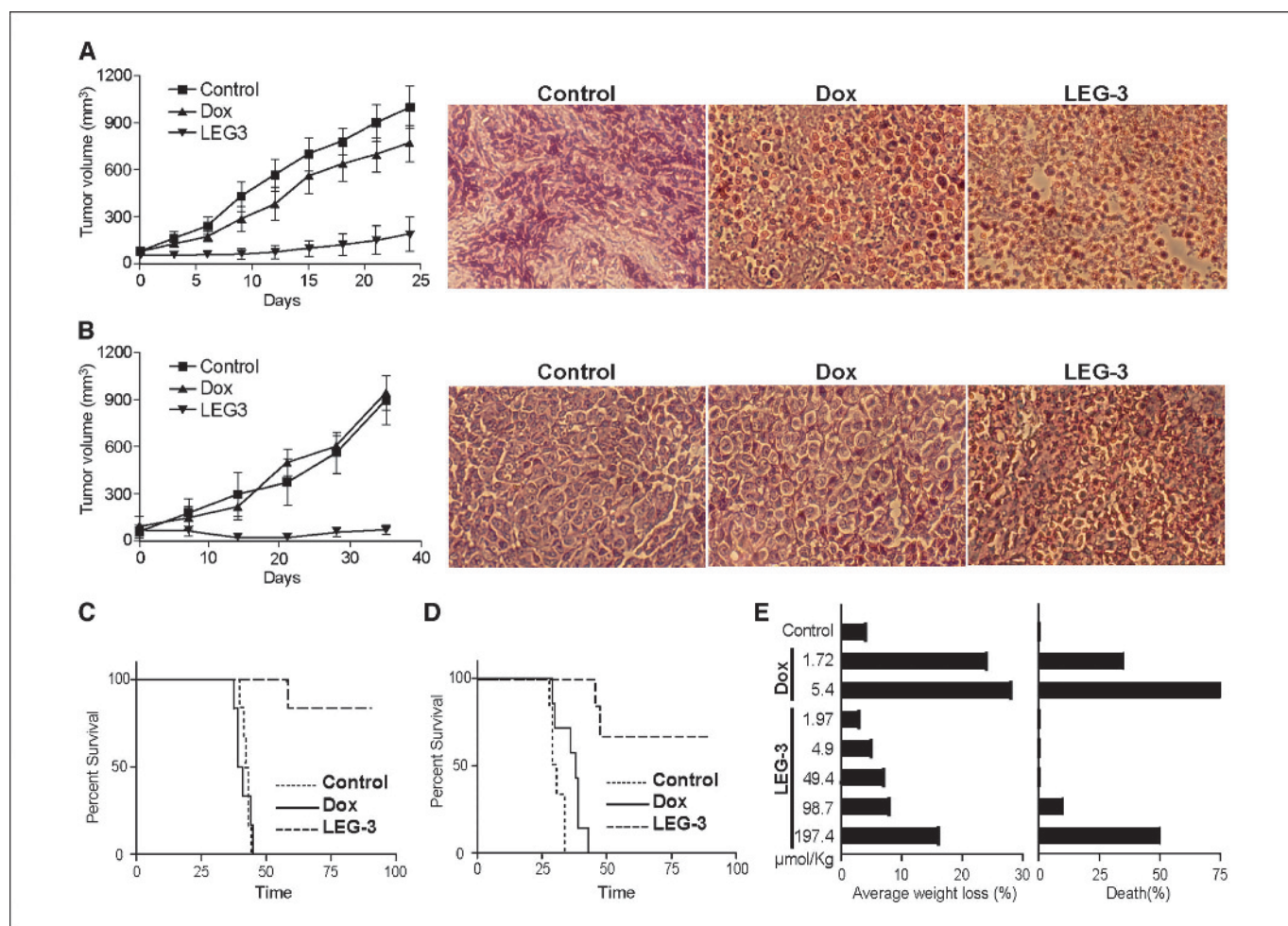


Figure 7. Efficacy of LEG-3 in human xenograft tumor models compared with doxorubicin. *A*, *in vivo* effects of LEG-3 (49.4 $\mu\text{mol/kg}$; $n = 8$) compared with doxorubicin (1.72 $\mu\text{mol/kg}$; $n = 8$) in the HT1080 human fibrosarcoma model and H&E-stained histologic analysis of control HT1080 tumor compared with tumors treated with doxorubicin and LEG-3. *B*, *in vivo* effect of LEG-3 (49.4 $\mu\text{mol/kg}$; $n = 8$) compared with doxorubicin (1.72 $\mu\text{mol/kg}$; $n = 8$) in the MDA-PCa-2b human prostate carcinoma xenograft model and H&E-stained MDA-PCa-2b human prostate carcinoma. *C*, survival analysis of HT1080 human fibrosarcoma models. *D*, survival analysis of mice bearing MDA-PCa-2b tumors after treatment. *E*, gross toxicity of tumor-bearing mice treated with doxorubicin and LEG-3.

as matrix metalloproteinases (38), cathepsins (39), and now legumain. Through these factors, tumor-associated macrophages stimulate or facilitate tumor angiogenesis, invasion, and growth, similar to the role of macrophages in wound healing. In fact, tumor cells circumvent the need to produce all the factors necessary for their growth and to establish a pseudo-organ, the solid tumor (11, 40), with the aid of tumor-derived molecules that can redirect tumor-associated macrophage activities to promote tumor survival and growth. The potent antitumor activity of LEG-3 *in vivo* may also be partly attributable to targeting endothelial cells and tumor-associated macrophages in the tumor microenvironment.

We here describe a tumor microenvironment-activated prodrug strategy for cancer therapy. LEG-3, the example, is activated by extracellular legumain in tumors leading to extensive tumor cell death and frequent complete tumor eradication, including drug-

resistant tumors. These data support the advance of this strategic class of therapeutics for development as a molecularly targeted cancer therapeutic. Given that this design may be adapted to other cytotoxic compounds, it represents an opportunity to advance cancer therapy.

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Overexpression of Legumain in Tumors Is Significant for Invasion/Metastasis and a Candidate Enzymatic Target for Prodrug Therapy¹

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ABSTRACT

Expression of legumain, a novel asparaginyl endopeptidase, in tumors was identified from gene expression profiling and tumor tissue array analysis. Legumain was demonstrated in membrane-associated vesicles concentrated at the invadopodia of tumor cells and on cell surfaces where it colocalized with integrins. Legumain was demonstrated to activate progelatinase A. Cells overexpressing legumain possessed increased migratory and invasive activity *in vitro* and adopted an invasive and metastatic phenotype *in vivo*, inferring significance of legumain in tumor invasion and metastasis. A prodrug strategy incorporating a legumain-cleavable peptide substrate onto doxorubicin was developed. The prototype compound, designated legubicin, exhibited reduced toxicity and was effectively tumoricidal *in vivo* in a murine colon carcinoma model.

INTRODUCTION

Functional genomics provides tools to identify genes and their products that may be of significance in the pathobiology of disease. Identification of molecular targets with therapeutic potential enables fundamental study of the molecular cell biology and the potential for rational drug development. In this study, gene expression profiling was undertaken in a murine colon carcinoma model, which revealed a high-level expression of legumain mRNA in tumors. Legumain is a recently identified lysosomal protease, a novel member of the C13 family of cysteine proteases (1). It is well conserved, present in plants, invertebrate parasites, as well as in mammals, and has a highly restricted specificity requiring an asparagine at the P1 site of substrates. The human legumain gene encodes a preproprotein of 433 amino acids. Murine legumain shares 83% homology with the human protein (2). Mammalian legumain has been implicated in processing of bacterial peptides and endogenous proteins for MHC class II presentation in the lysosomal/endosomal systems (3, 4). Recently, the human asparaginyl endopeptidase legumain has been identified as an inhibitor of osteoclast formation and is associated with bone resorption (5). Despite interest in legumain based on its novel substrate requirement and conservation through evolution, elucidation of its functional role in molecular cell biology and pathobiology is limited and association with tumor biology has not been suggested.

Here, we present evidence that legumain is overexpressed in the majority of human solid tumors. We demonstrate that legumain promotes cell migration, and overexpression is associated with enhanced tissue invasion and metastasis. Its unique functional properties and high-level expression in many human tumors lends itself as a potential candidate enzymatic target for prodrug activation and tumor eradication therapy.

The integrity of the amino group of doxorubicin is essential for function. It has been shown that doxorubicin tolerates the addition of a leucine residue at this site; however, incorporation of additional amino acids abolishes cytotoxic activity (6, 7). A prototype prodrug

was synthesized by addition to doxorubicin through a peptide bond of an asparaginyl endopeptidase substrate peptide. Upon exposure to legumain, the compound was converted to an active cytotoxic leucine-doxorubicin molecule. This compound had markedly reduced toxicity compared with doxorubicin and was effectively tumoricidal in a murine colon carcinoma model. It is proposed that legumain represents a new functional target for tumoricidal prodrug development and therapy.

MATERIALS AND METHODS

Reagents and Cell Lines. Rabbit polyclonal antisera against human legumain as well as 293 cells stably expressing human legumain were kindly provided by Dr. G. David Roodman (Department of Medicine and Hematology, University of Texas Health Science Center, San Antonio, TX). A legumain substrate peptide was synthesized by and purchased from Bachem (King of Prussia, PA). Doxorubicin was purchased from Sigma. Costar migration chambers were from Corning Incorporated (Corning, NY). Vitrogen was from Cohesion Technologies (Palo Alto, CA). Mouse monoclonal antibody specific for human integrin $\beta 1$ was from Dr. Richard L. Klemke (The Scripps Research Institute). DMEM was from Invitrogen (Carlsbad, CA). The CT26 murine colon carcinoma cell line was kindly provided by Dr. Ralph A. Reisfeld (The Scripps Research Institute). The 293 cells used to construct tetracycline-regulated cell lines expressing legumain were from Stratagene (La Jolla, CA). Multiple tumor tissue arrays were provided by Cooperative Human Tissue Network, National Cancer Institute.

Rapid Isolation of Tumor Endothelial Cells and mRNA Extraction. CD31 antibody-coated Dynabeads were prepared by mixing 300 μ l of bead suspension with 500 μ l of PBSA (PBS, 1% BSA). Biotinylated antimouse CD31 antibody (20 μ g) was added to the suspension, and association of antibody to beads was for 20 min at 4°C. The beads were washed three times with PBS to remove unbound antibody. CT26 tumors grown to ~1.5-cm greatest diameter were surgically removed cooled to 4°C for following steps, and the tumor minced into 1-mm³ bits with sterile scissors. The minced tumor was gently pressed through metal meshes and filtered through a 40- μ m Falcon cell strainer (Becton Dickinson, Franklin Lakes, NJ) to rapidly recover the single cell suspensions. Streptavidin-conjugated paramagnetic Dynabeads (DynaLink, Lake Success, NY) coated with biotinylated antimouse CD31 antibody (Mec 13.3; PharMingen, La Jolla, CA) were immediately added to the single cell suspensions. Capture by beads of CD31-positive cells was conducted at 4°C for 20 min with gentle agitation. Beads with bound CD31-positive cells were recovered with a magnetic trap column and washed three times with cold PBS. Unbound CD31-negative cells were collected separately and were recovered by centrifugation at 1000 rpm for 3 min. Both CD31-positive and CD31-negative cells were used for mRNA extraction (Qiagen mRNA direct kit). The concentration of mRNA was quantitated with RiboGreen RNA quantitation reagents (Molecular Probes, Eugene, OR).

Differential Gene Expression Profiling Using Restriction Fragment Differential Display. Five hundred ng of mRNA were used for differential profiling using the displayPROFILE method (Display Systems Biotech, Vista, CA). The mRNA was first used to synthesize double-stranded cDNA. The resultant double-stranded DNA was digested with Taq I, and adaptors were ligated onto the fragment ends. Display primer was used to PCR amplify the gene fragment profiles, which were then displayed on a 6% sequencing gel. Differentially displayed bands were cut from the sequencing gel and extracted with 50 μ l of water for 15 min in a boiling water bath. The fragments were reamplified with the same set of primers and then electrophoresed on 4% agarose gels. The amplified fragments were recovered from the gels and cloned into a pCRII vector by the Topo cloning method (Invitrogen). The vectors were

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then sequenced and BLAST searches performed with National Center for Biotechnology Information database to identify genes.

Histological and Immunohistochemical Analysis. Immunohistochemical staining was performed on both formalin-fixed and -unfixed frozen 5- μ m thick sections on poly-L-lysine slides. For endothelial identification, biotinylated rat antimouse CD31 monoclonal antibody (MEC 13.3) was used with fluorescein-conjugated streptavidin as the secondary reporting reagent. Rabbit anti-legumain antisera was prepared by immunization with purified human legumain produced in *Escherichia coli* (8). This antisera recognizes both mouse and rat legumain in frozen sections, as well as human legumain in formalin-fixed sections. For staining of legumain in both frozen and formalin-fixed sections, rabbit polyclonal anti-legumain antisera was used at 1:500 dilution, followed by biotinylated antirabbit IgG as the second antibody. The reaction was visualized with Texas-red conjugated streptavidin, and the slides were analyzed by laser scanning confocal microscope (Bio-Rad, Hercules, CA). For chromogenic staining, the rabbit polyclonal anti-legumain antibody was followed by a biotinylated goat antirabbit antisera (Vector, Burlingame, CA). Streptavidin-conjugated peroxidase was used and developed with the substrate BAD (Vector).

Western Blot. Proteins were dissolved in 2 \times SDS sample buffer for SDS-PAGE analysis using gradient Tris-glycine gels (8–16%). After electrophoresis, the proteins were transferred to nitrocellulose membranes and blocked with nonfat milk. The anti-legumain antisera was used as the first antibody and was incubated with membrane for 1 h (1:1,000 dilution). The blot was washed three times with PBS, incubated with streptavidin-peroxidase for 15 min, and developed by the enhanced chemiluminescence method (Sigma, St. Louis, MO).

Zymogram. Control 293 cells and legumain +293 cells were plated onto 96-well plates at 4000 cells/well. The cells were allowed to attach overnight, then were serum starved for 4 h. Zymogen forms of metalloproteinase 2 or 9 (Chemicon, Temecula, CA) were added at concentration of 0.1 μ g/well with 50 μ l of reaction buffer [39.5 mM citric acid, 121 mM Na₂HPO₄ (pH 5.8), 1 mM EDTA, and 0.8% Na₂Cl], and the reactions were continued for 10 min. The reactants were collected and mixed with an equal volume of SDS sample buffer and held at room temperature for 10 min then applied to a zymogram gel (10% Tris-glycine gel with 0.1% gelatin substrate). After electrophoresis, the gel was washed briefly and incubated with 2.5% (v/v) Triton X-100 at room temperature for 30 min with gentle agitation. Digestion of the incorporated gelatin by activated collagenase was conducted in buffer [50 mM Tris (pH 7.25), 200 mM NaCl, 10 mM CaCl₂, 0.05% Brij-35, and 0.02% NaN₃] overnight. The gel was stained with Coomassie Blue R250 (Novex, San Diego, CA), and the presence of a protease was readily observed as a clear band.

Cell Invasion and Mobility Assays. Cell migration and invasion assays were performed as described with modifications (9). Stock solutions (15 mg/ml) of Matrigel basement membrane matrix (Becton Dickinson, Bedford, MA) were stored at -80°C in 100- μ l aliquots. After thawing on ice, the stock was diluted 1:50 with cold serum-free culture media and immediately applied to each membrane insert (8- μ m pore) that formed the upper chambers of the multiwell invasion assay plate. The Matrigel was incubated overnight in a sterile laminar tissue culture hood. The membranes were hydrated for 2 h with 250 μ l of serum-free medium, and excess medium was removed by aspiration. Medium containing 10% FBS was added to the bottom of each well. A suspension of 10⁵ cells in 150 μ l of serum-free medium was added to the upper chamber and incubated for 12 h at 37°C, 5% CO₂. At the indicated times, the membrane inserts were removed from the plate, and the noninvading cells were removed from the upper surface. Membrane-associated cells were stained with 0.09% crystal violet for 30 min and washed twice with PBS. The invading cells were counted microscopically. Cell mobility assays were performed in a similar manner, except the membrane inserts were not coated with Matrigel, and duration was shortened. In some assays, protease inhibitors were added to the invasion chamber at the beginning of the incubation.

Prodrug Synthesis. *N*-(*t*-Butoxycarbonyl-L-alanyl-L-alanyl-L-asparaginy-L-leucyl)doxorubicin was synthesized as follows. To cold (0°C) solution of *t*-butoxycarbonyl-L-alanyl-L-alanyl-L-asparaginy-L-leucine (43 mg, 95 μ mol) and 4-methylmorpholine (20 μ l, 200 μ mol) in 5 ml of dimethylformamide was added *O*-benzotriazol-1-*N,N,N'*,*N'*-tetramethyluronium hexafluorophosphate (54 mg, 142.5 μ mol). After 10 min, doxorubicin hydrochloride (50 mg, 86 μ mol) was added, and the mixture was stirred for 2 h at room temperature in the dark. The solution was diluted with 30 ml of EtOAc and washed with

water. The solvent was evaporated, and solids were dried over MgSO₄ and purified over silica gel using CHCl₃/methanol (90/10) while protected from light to yield 65 mg of compound 1 (75% yield). ¹H NMR (600 MHz, CD₃OD, δ): 0.82 (d, [³H], *J* = 6.1), 0.88 (d, [³H], *J* = 6.6), 1.28–1.35 (m, 9H), 1.43 (s, 9H), 1.59–1.74 (m, 4H), 2.05 (m, 1H), 2.17 (m, 1H), 2.36 (d, 1H, *J* = 14.5), 2.67 (m, 1H), 2.79 (m, 1H), 2.91 (d, 1H, *J* = 18.0), 3.04 (d, 1H, *J* = 18.0), 3.62 (m, 1H), 4.01–4.04 (m, 4H), 4.11 (m, 1H), 4.22–4.32 (m, [³H]), 4.59 (dd, 1H, *J* = 5.9, 7.2), 4.74 (d, 2H, *J* = 4.4), 5.08 (s, 1H), 5.39 (d, 1H, *J* = 3.1), 7.51 (d, 1H, *J* = 8.8), 7.78 (dd, 1H, *J* = 7.9, 7.9), 7.86 (d, 1H, *J* = 7.5). Preparation high-resolution mass spectrometry (matrix-assisted desorption/ionization) calculated for C₄₈H₆₄N₆O₁₈ [M + Na]⁺ is 1035.4169 and found is 1035.4234. The compounds were purified by semipreparative high-performance liquid chromatography.

Cytotoxic Assays. The WST-1³ cell proliferation reagent (Roche Molecular Biochemicals, Indianapolis, IN) was used to determine cell proliferation by quantitation of cellular metabolic activity. Control 293 cells and legumain⁺ 293 cells were cultivated in microtiter plates (5 \times 10³ cells/well in 100 μ l) and were incubated with serial concentrations of legumain or doxorubicin for 48 h. Subsequently, 10 μ l of WST-1 solution (1 mg/ml WST-1, 25 μ M methylidibenzopyrazine methyl sulfate) were added/well, and mixtures were incubated for an additional 4 h. The tetrazolium salt WST-1 was cleaved by the mitochondrial succinate-tetrazolium-reductase system to formazan in cells, which directly correlates to the number of metabolically viable cells in the culture. The amount of formazan salt was quantified in three replicates by absorbance at 450 nm using a microplate reader (Molecular Devices, Palo Alto, CA). All results were derived from replicate experiments with similar results.

Animals Models. The CT26 syngeneic murine colon carcinoma model was generated and maintained in The Scripps Research Institute animal facility. Balb/C mice ages 4–6 weeks from the breeding colony were inoculated with 500,000 syngeneic CT26 tumor cells/site s.c. on the back. Treatment was initiated when the tumors reached 4 mm in diameter through bolus i.p. injection of the indicated reagents. Treatment was repeated at 2-day intervals. The human 293 tumor models were generated in WEHI nude mice (The Scripps Research Institute breeding colony). Either legumain⁺ 293 cells or control 293 cells (10⁶ cells/site) were inoculated s.q. on the back. Tumor growth and physical signs were monitored daily, including any gross evidence of tumor necrosis, local tumor ulceration, as well as evidence of toxicity, including mobility, response to stimulus, eating, and weight of each animal. These procedures have been reviewed and approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute. The work was conducted in The Scripps Research Institute facilities, which are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. The Scripps Research Institute maintains an assurance with the Public Health Service, is registered with the United States Department of Agriculture, and is in compliance with all regulations relating to animal care and welfare.

Statistical Analysis. Statistical significance of data were determined by the two-tailed Student's *t* test.

RESULTS

Overexpression of Legumain in Solid Tumors. Using restriction fragment differential display (10–13), we found that the cysteine protease legumain is highly expressed *in vivo* in the CT26 murine colon carcinoma. Immunohistochemical study of the CT26 tumor indicated that legumain is expressed by both tumor cells and frequently tumor associated endothelial cells, both intracellular and on the cell surface (Fig. 1A). Legumain overexpression in mouse tumors was confirmed by Western blot analysis of a panel of mouse tumors but is also expressed by some normal mouse tissues (Fig. 1B). Legumain expression is not detected in the CT26 cell line in culture that was used to generate the syngeneic mouse colon carcinoma model and is negative in other tumor cell lines in culture that we have tested. The remarkable up-regulation during tumor development *in vivo* infers an

³ The abbreviations used are: WST-1, (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disfonate]; TIMP-2, tissue inhibitor of metalloproteinase 2; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

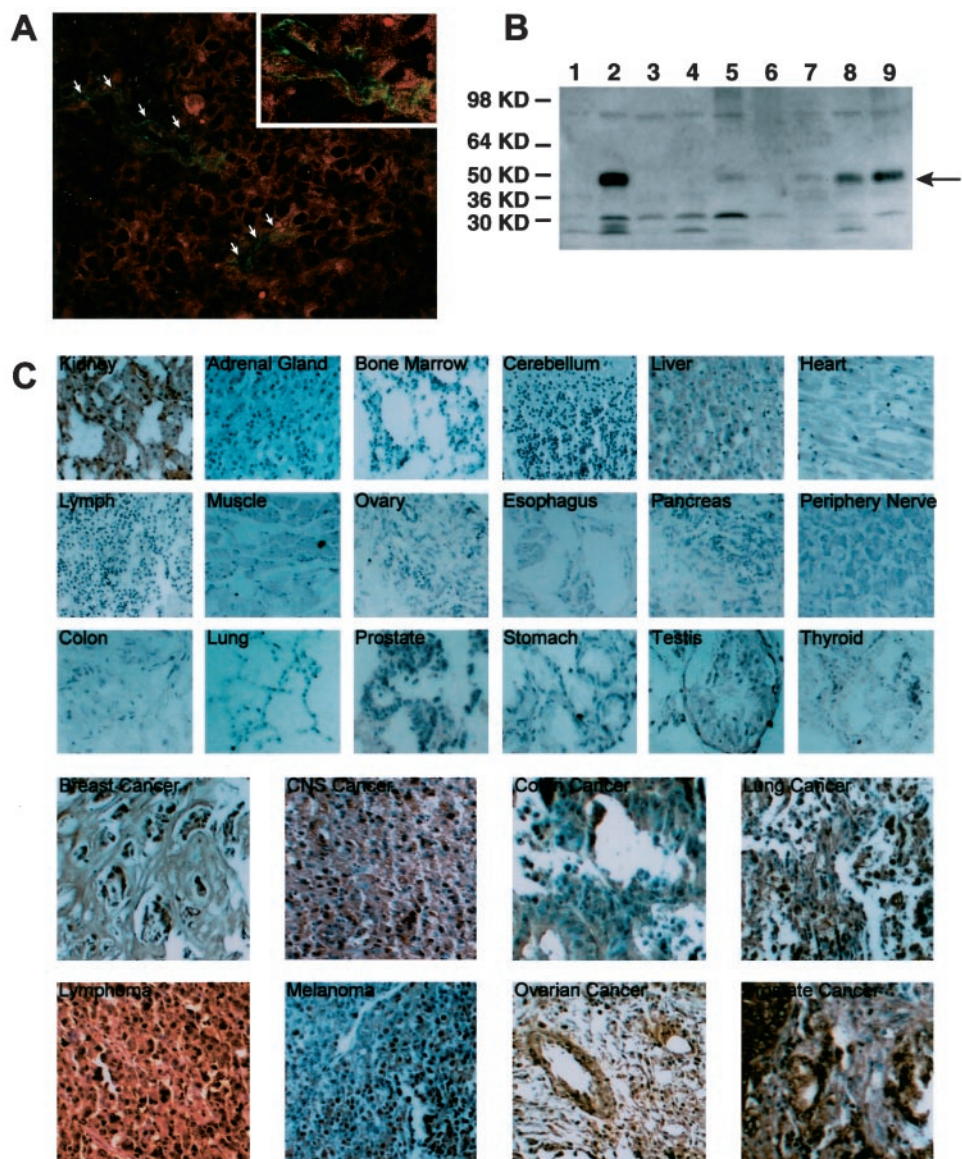


Fig. 1. Legumain is overexpressed in tumors. *A*, doubly stained section of CT26 mouse colon cancer. Legumain is red, and CD31⁺ endothelial cells are green (magnification, $\times 600$). Legumain expression is high in tumor cells and as well as endothelial cells. Legumain appears largely to be in membranous vesicles consistent with a distribution of endosomes/lysosomes. Legumain is also positive on the surface of tumor cells and endothelial cells (arrows). *B*, legumain expression profile by Western blot analysis. Lanes 1–9 are brain, tumor, lung, heart, muscle, intestine, spleen, liver, and kidney, respectively. Legumain expression is high in tumor. Legumain expression in normal tissues is highest in kidney, followed by liver and spleen. *C*, legumain protein positivity demonstrated in normal human tissues (magnifications, $\times 200$) and tumor specimens (magnification, $\times 400$) by anti-legumain antisera.

in vivo environmental response. Legumain appears to be a stress-responsive gene, although not detectable in cultured cells under typical tissue culture conditions, its expression was markedly elevated in cells subjected to environmental stress such as serum starvation or *in vivo* growth.

To characterize legumain expression in normal human tissues and tumors, we analyzed human tumor tissue arrays immunohistochemically with anti-legumain antisera (Fig. 1C). Whereas, in normal tissues legumain expression is sparse, highest in the kidney, presence in liver and spleen was detectable, agreeing with published results (1, 14). Notably, legumain was highly expressed in the majority of human tumor tissue panels analyzed, which encompassed a wide variety of solid tumors (Table 1). Expression was highest for prostate carcinomas and positive for most specimens from breast and colon carcinomas, and all central nervous system malignancies were positive.

Cellular Distribution of Legumain. Legumain is most abundantly visualized associated with intracellular membranous vesicles (Fig. 2A), consistent with the proposed function as a lysosomal protease. The delivery of membranous vesicles containing proteases, adhesion molecules, and actin-binding proteins toward the leading edge of migratory cells has been implicated in cell locomotion (15,

16). The legumain positive membranous vesicles were often concentrated at the invadopodia of tumor cells (Fig. 2B). However, we also observed it in apparent association with cell surfaces (Fig. 2C), as well as with the actin cortex (Fig. 2D). Double staining of legumain⁺ 293 cells with antibody against integrin $\beta 1$ and anti-legumain antibody disclosed the presence of legumain inside cells in a granular organelle resembling aggregated lysosomes and also on the cell surface colocalized with $\beta 1$ integrins (Fig. 2E). The potential binding of legumain

Table 1 Legumain detection in human solid tumors

| Carcinoma type | Number analyzed | Number positive | Percentage positive | Degree of positivity |
|-------------------------------|-----------------|-----------------|---------------------|----------------------|
| Breast carcinoma | 43 | 43 | 100% | +++ |
| Colon carcinoma | 34 | 32 | 95% | +++ |
| Lung carcinoma | 24 | 14 | 58% | +++ |
| Prostate carcinoma | 56 | 42 | 75% | ++++ |
| Ovarian carcinoma | 23 | 17 | 73% | ++ |
| Central nervous system tumors | 8 | 8 | 100% | ++ |
| Lymphoma | 14 | 8 | 57% | + |
| Melanoma | 12 | 5 | 41% | + |

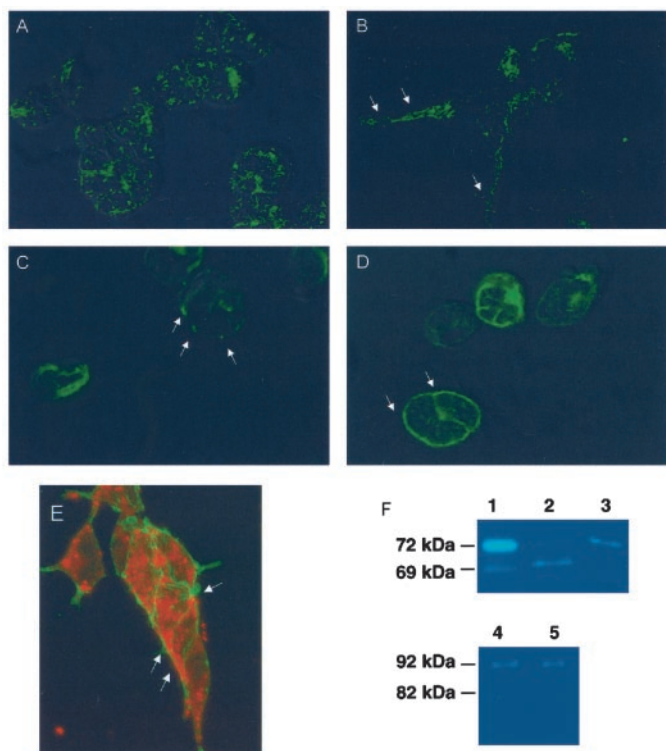


Fig. 2. Cellular distribution of legumain and activity. *A*, legumain is detected (green) in intracellular vesicles, and (*B*) prominently associated with the invadopodia of migrating tumor cells. *C*, legumain is also observed on cell surface of serum-starved BEND3 cells, and with (*D*) the actin cortex of same cells. *E*, doubly stained legumain⁺ 293 cells demonstration of legumain in red and integrin $\beta 1$ in green. Legumain appears in a granular organelle that resemble aggregated lysosomes as well as on the cell surface colocalizing with $\beta 1$ integrins (arrow). Magnification, $\times 1000$. *F*, conversion of M_r 72,000 progelatinase A to M_r 62,000 active enzyme by legumain. Activation was minimal in reaction with control 293 cells (Lane 1), but the majority of zymogen was converted to active when reacted with legumain⁺ 293 cells (Lane 2), and activation can be fully inhibited with presence of cystatin (Lane 3). Legumain was not active against progelatinase B (Lanes 4 and 5 are progelatinase B with 293 cells and legumain⁺ 293 cells, respectively).

to $\beta 1$ integrins is provocative considering the Arg-Gly-Asp sequence motif present in legumain, a motif that might facilitate legumain association with cell surface integrins.

Activation of Progelatinase A by Legumain. Tumor cell surface-associated proteases may degrade extracellular matrix proteins (gelatinase A and cathepsins). Legumain does not degrade gelatin directly when analyzed in a gelatin zymogram (data not shown). The activation of progelatinase A requires cleavage of an asparaginyl bond (17). We examined whether cell surface bound legumain can convert the M_r 72,000 zymogen to the M_r 62,000 active enzyme. Cells expressing legumain as well as control cells were deposited in 96-well plates. After attachment and serum starvation, the culture media was removed. Zymogen forms of gelatinase A and B were incubated with the cells for 10 min, and the reaction products were analyzed by zymography. Generation of the M_r 62,000 active enzyme was observed for cells expressing legumain, and the activation was inhibited by cystatin (Fig. 2*F*). Recently, activation of zymogen gelatinase A by legumain has been reported (18). No effect of legumain on zymogen gelatinase B was detected (Fig. 2*F*).

Legumain Expression Promotes Cell Migration and Invasion. We evaluated the effect of legumain expression on cell migration and invasion. In an *in vitro* migration assay, legumain⁺ 293 cells exhibited an increased migration in comparison with wild-type 293 cells, and the enhanced migration was inhibited by cystatin, a known inhibitor of legumain protease function, weakly by TIMP-2 protein but not by E64

(Fig. 3*A*). Next, we evaluated control 293 cells and legumain⁺ 293 cells in a modified Boyden chamber invasion assay. Legumain⁺ cells exhibited increased invasion of extracellular matrix, which was inhibited by cystatin (Fig. 3*B*) but to only a limited extent by TIMP-2. Again E64 was without effect. These experiments were repeated with a 293 cell line in which the transcription of legumain was conditionally regulated by tetracycline with comparable results (data not shown).

Legumain Expression Correlates with Tumor Invasion and Metastasis. To explore the effects of legumain expression *in vivo*, legumain⁺ 293 cells and control 293 cells were injected s.c. on the back of WEHI nude mice. Tumors appeared after 2–3 weeks, and the initial rates of primary tumor growth was comparable. There was prominent legumain expression in the legumain⁺ 293 tumors. Weak but positive legumain expression was detectable in control 293 tumors despite lack of legumain expression by these cells in culture, similar to the observations for the CT26 colon carcinoma cells. Histological analysis of more advanced tumors suggested a lower rate of apoptosis for legumain⁺ 293 tumors compared with control 293 tumors. Legumain⁺ 293 tumors frequently metastasized *in vivo* in contrast to control 293 tumors. At necropsy, 50% of legumain⁺ 293 tumor-bearing mice had metastatic nodules in distant organs (Fig. 4*A*), predominantly lung and liver, whereas none were observed for control 293 tumors. The increased invasion and metastasis associated with legumain overexpression is consistent with legumain-facilitated tumor metastasis and progression. The more invasive legumain⁺ 293 tumors frequently invaded muscles and frequently lacked the well-defined pseudoencapsulation observed with control 293 tumors (Fig. 4, *B* and *C*). This more invasive tumor behavior was evident in early as well as later stage tumors.

Model Prodrug Activation by Legumain. The functional capacity of tumor cell-associated legumain was explored based on the novel asparaginyl-specific endopeptidase activity of legumain. The amino group of doxorubicin is critical for function, however, a leucine residue can be added with retention of cytotoxicity. Other amino acids or longer peptide extensions abolish activity of the derived compound. An experimental prodrug analogue, N-(*t*-Butoxycarbonyl-L-alanyl-L-

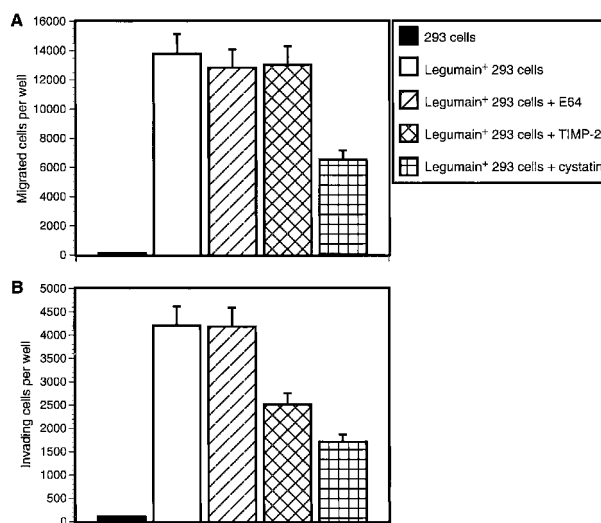


Fig. 3. Legumain expression promotes cell migration and invasion. *A*, migration of legumain⁺ 293 cells was markedly greater than control 293 cells. The enhanced cell migration was partially inhibited by cystatin but not by TIMP-2 or E64. *B*, expression of legumain enhanced 293 cell invasion across a Matrigel barrier compared with control 293 cells. The invasive activity was partially inhibited by cystatin and TIMP-2 but was not affected by the presence of E64. Each bar represented the mean \pm SE of three independent wells, and the experiments were repeated at least three times with similar results. $P < 0.001$.

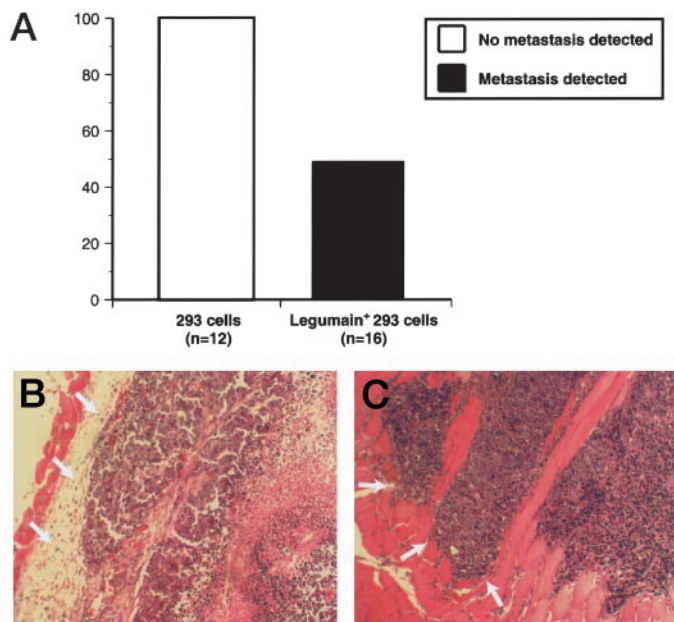


Fig. 4. Legumain enhances tumor invasion and metastasis *in vivo*. **A**, distant metastasis were detected in 50% of WEHI nude mice inoculated with 293 cells overexpressing legumain versus mice inoculated with wild-type 293 cells. **B**, tumor generated in WEHI nude mice with legumain⁺ 293 cells. **C**, tumor generated by control 293 cells. Note the pseudoencapsulation seen in the typical control 293 cell tumors (arrows) was lacking in legumain⁺ 293 cell tumors, and legumain⁺ 293 tumor invasion of muscle was frequent (arrows). Magnification, $\times 200$.

alanyl-L-asparaginyl-L-leucyl)doxorubicin, was synthesized by addition of an asparaginyl endopeptidase substrate peptide Boc-Ala-Ala-Asn-Leu to the amino group of doxorubicin through a peptide bond at COOH terminus of leucine. Upon cleavage by legumain, the prodrug will be converted to a leucine-dox molecule, thereby regaining cytotoxic function. In addition, the presence of the Boc at the NH₂ terminus prevents aminopeptidase hydrolysis of the peptidyl component. We designated this Legubicin (Fig. 5A).

First, the cytotoxic activity of legubicin upon activation by legumain was analyzed *in vitro* using legumain⁺ 293 cells and control 293 cells (Fig. 5B). The effect of doxorubicin on both 293 cell types was similar, with legumain⁺ cells only slightly more resistant to doxorubicin. In contrast, the cytotoxic effect of legubicin on control 293 cells was <1% of that of doxorubicin, indicating peptide conjugation had abolished the cytotoxic effect of the doxorubicin. The dose-responsive curve of legubicin on control 293 cells was parallel to that of doxorubicin on both 293 cells, suggesting that the residual cytotoxicity may result from slight (~1%) doxorubicin contamination. In contrast, a profound cytotoxic effect of legubicin was observed for legumain⁺ 293 cells. The dose-response curve of legumain⁺ cells challenged with legubicin differed from that for these cells exposed to doxorubicin.

Tumoricidal Effect of Legubicin *in Vivo*. The *in vivo* effects of legubicin on normal and tumor-bearing hosts, and efficacy in tumor eradication was investigated using the CT26 murine syngeneic colon carcinoma model. Legubicin was very well tolerated in mice with much reduced toxicity compared with doxorubicin. i.p. injection of legubicin at 5 mg/kg three times at 2-day intervals induced complete growth arrest of the tumors (Fig. 6A, top and middle panel) with little evidence of toxicity, as most readily evidenced by the absence of weight loss (Fig. 6A, bottom panel). In contrast, doxorubicin failed to produce similar antitumor efficacy at doses approaching its maximum-tolerable dose. When doxorubicin was administered by the same protocol and dosage as for legubicin toxicity was fatal (Fig. 6A, top and middle panel).

A single injection of 5 mg/kg legubicin histologically induced more profound tumoricidal effects than animals given a comparable dose of doxorubicin (Fig. 6, B and C). TUNEL assay analysis of tumor tissues revealed a higher apoptotic index for legubicin than for doxorubicin treatment (Fig. 6, D and E). Surprisingly, in organs that do express legumain such as kidney and liver, no injury was evident (data not shown). These observation indicate that legubicin has significantly improved safety and therapeutic indices compared with doxorubicin.

DISCUSSION

Positional gene expression profiling of tumor tissue holds promise of advancing knowledge of the molecular pathobiology of neoplasia and molecularly targeted cancer therapy in the postgenomic era. Motivated by the discovery of legumain overexpression in tumors, we sought to investigate the potential role of this novel protease in tumor biology and, if appropriate, to design therapy that could target such tumors. We found legumain to be highly expressed by most human tumors surveyed. A high percentage of breast carcinomas, colon carcinomas, and central nerve system neoplasms strongly expressed legumain, with the highest expression found in prostate tumors, whereas legumain was weakly expressed or not observed in the normal tissues of tumor derivation. It was negative for the cell lines in culture that were used to generate the *in vivo* tumors, although positive *in vivo*, indicative of induction of gene expression by the *in vivo* tumor environment. Proteases have been implicated in many aspects of tumor cell biology (19). Thus, a protease that is highly expressed by tumor cells or tumor vascular endothelial cells might contribute to tumor cell progression through processing of signaling molecules and their receptors, thereby influencing cellular responses. Such effects might also result in diminished apoptosis (20), thereby enhancing tumor growth.

In this study, we present evidence for, not only atypical expression, but also the participation of legumain in effector functions and as an apparent regulator of cellular behavior in migration and tissue inva-

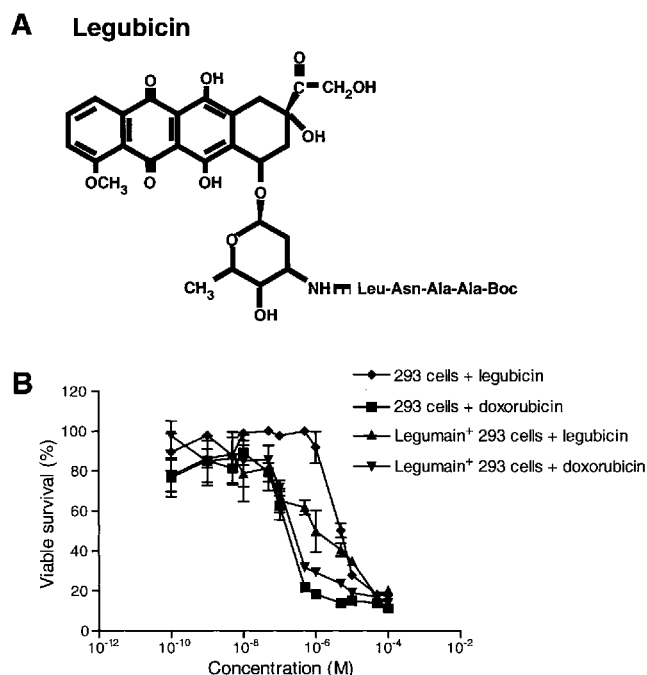
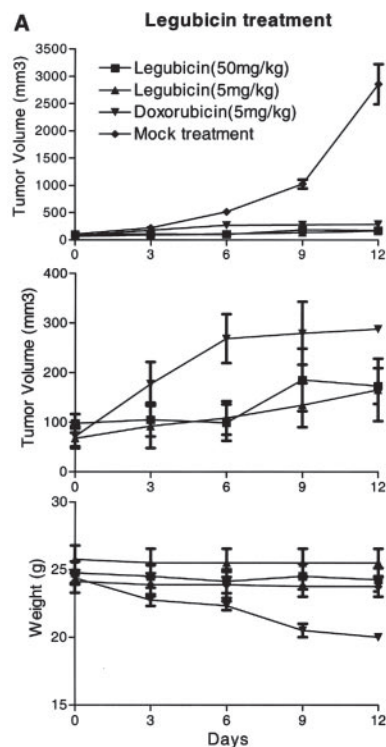


Fig. 5. Asparaginyl endopeptidase activated prodrug legubicin. **A**, chemical structure of legubicin. **B**, cytotoxic assays of legubicin and doxorubicin in legumain⁺ 293 cells and control 293 cells. Cytotoxic activity of legubicin is much higher on legumain⁺ cells consistent with prodrug activation by these cells.

Fig. 6. Tumoricidal effect of legumain CT26 colon carcinoma *in vivo*. A, *in vivo* effect of legumain on CT26 colon carcinoma. Three i.p. injections at both 5 and 50 mg/kg were administered with 2-day intervals. Legumain-arrested tumor growth and tumor eradication was achieved (top and middle panel) with little evidence of toxicity as represented by weight loss (bottom panel). In contrast, doxorubicin caused death of host animals at 5 mg/kg. The middle panel is a graph of the same experiment presented in the top panel without data from the mock-treated control group. B, H&E staining of tumor treated with legumain (magnification, $\times 1320$), and (C) H&E staining of tumor treated with equivalent dose of doxorubicin (magnification, $\times 1320$). D, TUNEL assays of tumor specimens revealed that tumors treated with legumain (magnification, $\times 400$) have higher apoptotic index than that treated with doxorubicin (magnification, $\times 400$; E).



sion. Cells that highly express legumain-exhibited enhanced migratory and invasive properties. A correlation between tumor invasion and metastasis with the presence of cysteine endopeptidases (particularly cathepsins B and L) has been observed (21). Hydrolysis of asparaginyl bonds is prominent in the posttranslational processing of cathepsin B, D, and H (1, 14, 22). Legumain might activate local cysteine protease zymogens to their active two-chain protease forms. In addition to the established plasminogen/plasmin system and the metalloproteinase system, a cysteine protease cascade may represent an additional tumor invasion/metastasis cascade. We as well as others have provided evidence that legumain activates the gelatinase A zymogen, an important mediator of extracellular matrix degradation. The activation mechanism of gelatinase A by legumain differs from that involved with the membrane type matrix metalloproteinases (23). This may be important for tumor cell adaptation to a more invasive and metastatic phenotype. Legumain-promoted cell migration and invasion can be partially inhibited by cystatin and TIMP-2, which suggests that there may be a set of derivative events and downstream effector enzymes. The inhibition of mammalian legumain by cystatin is attributable to a novel second reactive site (24). Another cysteine inhibitor E64 has no effect on legumain and excludes typical papain family cysteine proteases, which are characteristically susceptible to E64 inhibition. Analysis by site-directed mutagenesis of the catalytic residues of mammalian legumain implicates a catalytic dyad with the motif His-Gly-spacer-Ala-Cys. The presence of this motif is also found in the catalytic sites of the caspases, the aspartate-specific endopeptidases central to the process of apoptosis in animal cells, as well as in the families of clostripain and gingipain which are arginyl/lysyl endopeptidases of pathogenic bacteria. Therefore, these four families of proteases may be evolutionarily related and share similar protein folding. In this respect, legumain is notably distinct from other lysosomal cysteine proteases, and its catalytic activity is rather unique. It is the only asparaginyl endopeptidase identified to date, its conservation through evolution and unique enzymatic activity support selection for a significant biological function.

Animal tumor models generated with cells overexpressing legumain demonstrated an *in vivo* behavior that is vigorous with more invasive growth and metastasis. This phenotype is proposed to result from the proteolytic function of legumain to activate other protease zymogens. The inhibitory effect of cystatins on tumor cells (25, 26) is consistent with the involvement of legumain and perhaps other cysteine proteases in tumor invasion and metastasis. Whether the tumor arrest effect is mediated through inhibition of legumain catalytic activity or other cysteine proteases is presently unknown. Tumor invasion and metastasis are critical determinants of cancer lethality, linked to 90% of human cancer deaths (27). Invasion and metastasis are considered as associated properties of tumor cells because they use similar processes involving physical attachment of cells to their environment through cell adhesion molecules and activation of extracellular proteases (20). Increased expression of proteases and down-regulation of protease inhibitors is commonly observed in tumors (28, 29). Notably, cell surface proteases are often associated with invasive and metastatic tumor cells (19). Some proteases are linked to other properties of tumors such as angiogenesis (30) and growth signaling (31) as perhaps with legumain.

Protease zymogens are dependent on limited proteolytic activation for conversion to the functional state. Protease cascades are characteristic of many biological pathways such as the coagulation, apoptosis, and complement cascades. Similar cascades appear to be involved in tumor invasion and metastasis. Characterization of the later is complicated by the diversity of neoplasms. However, comprehensive profiling of protease expression and function may advance understanding of tumor invasion and metastasis.

Some metalloproteinase inhibitors have demonstrated tumor stasis in animal models. Similarly, legumain may represent a target for inhibition of growth and metastasis based on its up-regulation associated with tumor growth and unique restricted specificity. Legumain functions both extracellular and intracellular, therefore, a cell permeable inhibitor might extend the efficacy observed with cystatin be-

cause the latter is cell impermeable and has shown limited inhibition of *in vitro* cell migration and invasion.

We observed that tumor cells with higher legumain levels appear more resistant to apoptosis. Although the precise molecular pathway has yet to be defined for this effect, lysosomal proteases are known to participate as effector enzymes in apoptosis (32, 33). In another context, others have been observed to inhibit apoptosis (34). Thus, the subcellular localization of legumain may determine its targets and thereby its effects on the apoptosis cascades.

The high level of legumain expression by tumor cells coupled with its unusual and highly specific substrate requirement for catalytic function makes it an attractive candidate for prodrug conversion in a therapeutic mode. Current cancer chemotherapeutic agents have significant undesirable cytotoxicity. A promising approach to increase selectivity is to exploit enzymes more highly expressed by tumor cells to achieve local prodrug activation to the active compound. Peptide conjugates of doxorubicin designed for activation by plasmin (35, 36) and cathepsins (37–39) have been described. However, they are relatively deficient in target selectivity because plasmin generation is not tumor selective. The prodrug example in the present study was synthesized by incorporating a peptide extension to the amino group of doxorubicin. This compound, designated legubicin, was analyzed for cytotoxicity on cells not expressing legumain where it was <1% as toxic as doxorubicin. However, on cells expressing legumain, it was profoundly cytotoxic consistent with conversion to leucine-doxorubicin. i.p. administration of legubicin at 5 mg/kg resulted in complete arrest of tumor growth without identifiable toxicity such as weight loss in contrast to doxorubicin-treated mice. Legubicin administration produced profound tumor cell apoptosis as indicated by TUNEL assay. To our surprise, in organs containing cells that normally express legumain such as kidney and liver, no injury was evident. These data indicated that legumain activation of this prodrug may require conditions not present in normal tissue. First, prodrug activation may be carried out by secreted or cell surface-associated legumain, whereas legumain appears to be localized in lysosomal vesicles in normal tissues. Second, legumain requires an acidic environment for optimal catalytic activity, which may not be present in normal tissues. Our data suggest legubicin has an improved therapeutic index compared with its parent doxorubicin. Whereas clinical use of doxorubicin is limited by its toxicity, a prodrug that preserves activity but with reduced toxicity is an attractive alternative.

In summary, we have demonstrated unsuspected high-level expression of legumain, a novel asparaginyl endopeptidase, in a wide variety of tumors, murine and human. Legumain expression appears to be associated with increased tumor invasion and metastasis, possibly through increased extracellular matrix degradation, resulting from activation of zymogens such as progelatinase A and also to reduce tumor cell apoptosis. The legumain-activated prodrug legubicin, exhibited reduced toxicity relative to doxorubicin, and was effectively tumoricidal *in vivo*. This study testifies to the strength of functional genomics in guiding rational drug design.

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Targeting tumor-associated macrophages as a novel strategy against breast cancer

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Tumor-associated macrophages (TAMs) are associated with tumor progression and metastasis. Here, we demonstrate for the first time that legumain, a member of the asparaginyl endopeptidase family functioning as a stress protein, overexpressed by TAMs, provides an ideal target molecule. In fact, a legumain-based DNA vaccine served as a tool to prove this point, as it induced a robust CD8⁺ T cell response against TAMs, which dramatically reduced their density in tumor tissues and resulted in a marked decrease in proangiogenic factors released by TAMs such as TGF- β , TNF- α , MMP-9, and VEGF. This, in turn, led to a suppression of both tumor angiogenesis and tumor growth and metastasis. Importantly, the success of this strategy was demonstrated in murine models of metastatic breast, colon, and non-small cell lung cancers, where 75% of vaccinated mice survived lethal tumor cell challenges and 62% were completely free of metastases. In conclusion, decreasing the number of TAMs in the tumor stroma effectively altered the tumor microenvironment involved in tumor angiogenesis and progression to markedly suppress tumor growth and metastasis. Gaining better insights into the mechanisms required for an effective intervention in tumor growth and metastasis may ultimately lead to new therapeutic targets and better anticancer strategies.

Introduction

A novel antitumor strategy is immunization against molecules overexpressed by tumor-associated macrophages (TAMs) and thereby remodel the tumor microenvironment that attracts these macrophages and mediates their function (1, 2). TAMs consist primarily of a polarized M2 (F4/80⁺/CD206⁺) macrophage population with little cytotoxicity for tumor cells because of their limited production of NO and proinflammatory cytokines (3). TAMs also possess poor antigen-presenting capability and effectively suppress T cell activation. In fact, these macrophages of M2 phenotype actually promote tumor cell proliferation and metastasis by secreting a wide range of growth and proangiogenic factors as well as metalloproteinases and by their involvement in signaling circuits that regulate the function of fibroblasts in the tumor stroma (4). In recent studies, anti-TAM effects induced by small molecule inhibitors contributed to tumor suppression (5, 6). For example, the antineoplastic agent Yondelis has a selective cytotoxic effect on TAMs, thereby significantly reducing their production of IL-6 and CCL2, which, in turn, contribute to growth suppression of inflammation-associated human tumors (7). Another such example is provided by a biphosphonate compound, zoledronic acid, that suppresses MMP-9 secretion by TAMs, thereby inhibiting tumor metalloproteinase activity and diminishing the association of VEGF with its tyrosine kinase receptors on proliferating endothelial cells (8). In a different experimental model, the chemokine CCL5 was shown to be key in the recruitment of TAMs, and an antagonist of this chemokine reduced the tumor infiltrate and slowed tumor growth (9). Hence, although the therapeutic targeting of TAMs is still in its infancy, initial clinical results are encourag-

ing, as they suggest that targeting TAMs may complement more conventional cancer treatment regimens.

Legumain is a novel evolutionary offshoot of the C13 family of cysteine proteases (10). It is well conserved in plants and mammals, including humans. It was first identified in plants as a processing enzyme of storage proteins during seed germination (11, 12) and was subsequently identified in parasites and then in mammals (13). Legumain is a robust acidic cysteine endopeptidase with remarkably restricted specificity, absolutely requiring an asparagine at the P1 site of its substrate sequence (13). The selection of legumain as a target for tumor therapy is based on the fact that the gene encoding this asparaginyl endopeptidase was found to be highly upregulated in many murine and human tumor tissues (1, 14, 15) but absent or present only at very low levels in all normal tissues from which such tumors arise. Importantly, overexpression of legumain occurs under such stress conditions as tumor hypoxia, which leads to increased tumor progression, angiogenesis, and metastasis. In this regard, we recently discovered that legumain is heavily overexpressed by TAMs in murine breast tumor tissues by using gene expression profiling and immunohistochemistry. Importantly for our studies, TAMs have a particularly abundant expression in the tumor stroma (14) and express high levels of legumain in this tumor microenvironment. In contrast, classical macrophages of the M1 phenotype, which perform key immune-surveillance and antigen-presentation functions, do not express legumain. Consequently, targeting TAMs that overexpress legumain does not interfere with the biological functions of M1 macrophages, including cytotoxicity and antigen presentation (16–18).

Based on these findings, we hypothesized that targeting TAMs that overexpress legumain will reduce their density and thereby remodel the tumor microenvironment. This should lead to the downregulation of a wide variety of tumor growth factors, proangiogenic factors, and metalloproteinases released by these M2

Nonstandard abbreviations used: TAM, tumor-associated macrophage.

Conflict of interest: The authors have declared that no conflict of interest exists.

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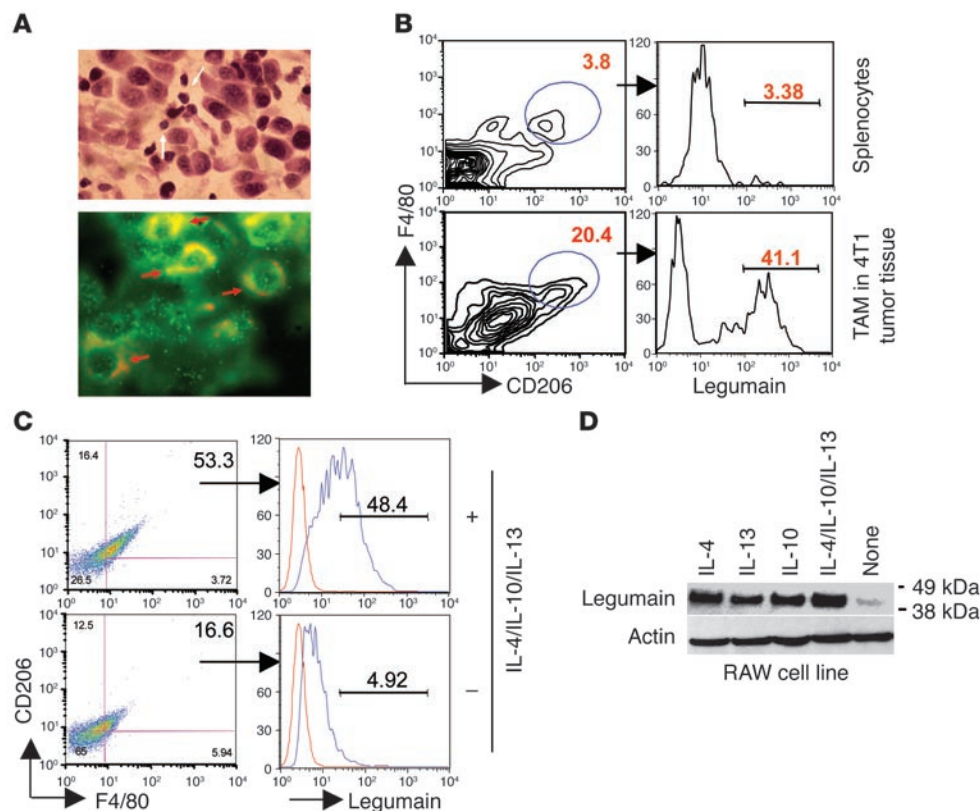


Figure 1

Legumain is highly expressed on TAMs in the tumor stroma. (A) Legumain expression on TAMs was clearly evident. Tumor-infiltrating macrophages were visualized by H&E staining, as indicated by arrows. Legumain expression is indicated by double staining with anti-legumain Ab (green) combined with anti-CD68⁺ Ab (red). Magnification, $\times 350$. (B) Increased legumain expression on TAMs was confirmed by flow cytometric analyses with double-positive populations of F4/80⁺/CD206⁺ M2 macrophages that were isolated from fresh tumor tissue. (C) Multiple-color flow cytometry demonstrated upregulation of the M2 macrophage marker CD206 on RAW cells after being cultured with IL-4, IL-10, and IL-13 (10 ng/ml). Furthermore, legumain was shown to be highly expressed on F4/80⁺/CD206⁺-positive RAW cells cultured with IL-4, IL-10, and IL-13 (upper panels) compared with wild-type RAW cells (lower panels). (D) Confirmation of legumain expression on RAW cells by Western blotting following stimulation with IL-4, IL-13, and IL-10, either singularly or combined.

macrophages and thereby decisively suppress angiogenesis of tumors as well as their growth and metastasis. To test our hypothesis, a legumain-based DNA vaccine served as a tool to eliminate TAMs in murine models of colon, breast, and lung tumor metastases.

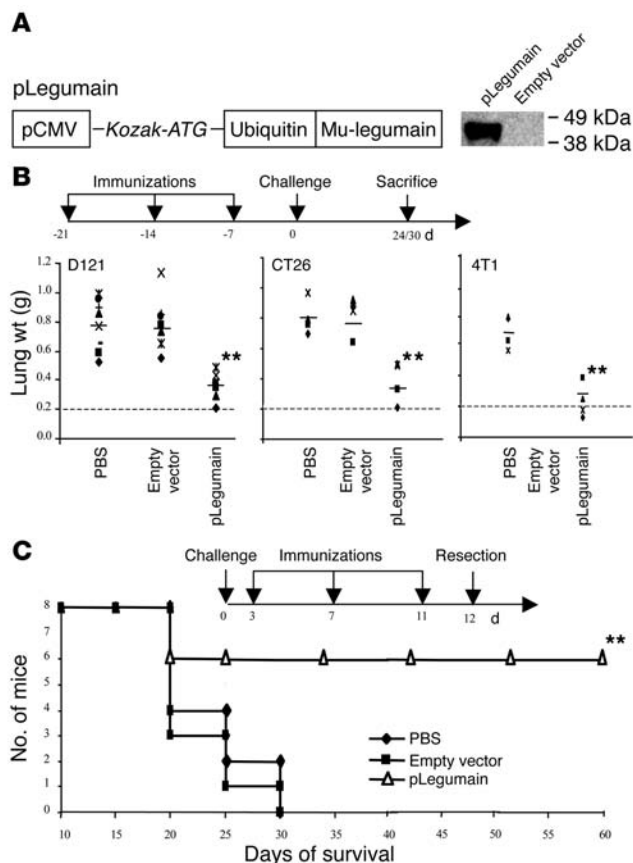
Results

Legumain serves as a target to kill TAMs overexpressed during tumor progression. It is well known that TAMs play a key role in tumor progression and metastasis (5). Therefore, targeting of these M2 macrophages represents a novel antitumor strategy. We initially identified legumain as a significant marker molecule of TAMs, since it was highly overexpressed on these cells in the tumor microenvironment and stroma. To this end, we isolated TAMs from murine 4T1 breast tumor tissue and demonstrated by flow cytometry that legumain was highly overexpressed on CD206 and F4/80 double-positive M2 macrophages, especially when compared with normal M1 macrophages in the spleen (Figure 1B). This result was also confirmed by immunohistochemical analyses, indicating that TAMs could be visualized by H&E staining, and legumain overexpression was further indicated by double stain-

ing with anti-legumain Ab (Figure 1A, green) combined with anti-CD68 Ab (red). These data demonstrate that infiltrating TAMs are a disproportionately large cell subpopulation in 4T1 tumor tissue and that legumain is a potentially effective target for killing TAMs.

Induction of legumain expression on TAMs by Th2 cytokines. In order to determine whether legumain expression on TAMs was induced by such Th2 cytokines as IL-4, IL-10, and IL-13, we cocultured a murine macrophage cell line, RAW, with these cytokines. This resulted in a significant increase in F4/80⁺/CD206⁺ expression by these RAW cells, concurrent with an upregulation of legumain (Figure 1C). These results were confirmed by Western blotting (Figure 1D). However, we found no evidence for legumain expression by tumor cell lines when cultured with these same cytokines (data not shown). These findings suggest that Th2 cytokines such as IL-4, IL-10, and IL-13 are released by tumor and other tumor stromal cells and accumulate in the tumor microenvironment, where they could potentially induce the proliferation and transformation from M1 macrophages to a population with an M2 phenotype that overexpresses legumain.

Targeting of TAMs suppresses tumor progression. Growth and metastases of tumors are highly coordinated with the presence of TAMs, and therefore targeting of this macrophage subpopulation leads to suppression of tumor growth and metastases. To test this hypothesis, we generated an expression vector for a DNA vaccine encoding legumain. Figure 2A schematically depicts the vector construction map based on the pCMV/myc/cyto vector backbone. The gene encoding legumain was fused to the C terminus of mutant polyubiquitin (pLegumain) to improve antigen processing in the proteasome (19), and the entire fragment was then inserted between the PstI and NotI restriction sites, while protein expression was demonstrated by Western blotting. We further tested our hypothesis that reducing the number of TAMs by our legumain-based DNA vaccine can effectively inhibit spontaneous 4T1 breast cancer metastases or experimental metastases of either D121 non-small cell lung or CT26 colon carcinomas. Thus, in a prophylactic setting, C57BL/6J mice were immunized 3 times with either PBS, empty vector, or pLegumain carried by attenuated *Salmonella typhimurium*. One week after the last immunization, these mice were challenged i.v. with 2×10^5 D121



non-small lung tumor cells, and 24 days thereafter experimental lung metastases were measured and analyzed. In the 2 control groups, the average lung weight was significantly greater than that of the vaccination group (Figure 2B). Similar results were obtained in the CT26 colon tumor model and 4T1 breast cancer model in syngeneic BALB/c mice (Figure 2B).

In a more demanding therapeutic setting, BALB/c mice were first challenged with 4T1 breast cancer cells and then immunized 3 times with the legumain-based DNA vaccine or an empty control vector. Twelve days after challenge with 4T1 tumor cells, the primary tumor was surgically excised, and the resulting life-span curve indicated that 75% (6/8) of the mice immunized with pLegumain survived for 3 months. In contrast, mice in the control groups all died within 1 month (Figure 2C). These data indicate that the legumain-based DNA vaccine effectively suppresses tumor cell growth and metastases in mouse models of 4T1 breast cancer, D121 non-small cell lung cancer, and CT26 colon carcinoma. Combined with surgery, this vaccine could indeed significantly extend the life span of mice by inhibiting tumor cell metastases in these very challenging therapeutic mouse tumor models.

Targeting legumain induces a specific CD8⁺ CTL response, decreasing TAM populations in the tumor stroma. Immunization against legumain induced a specific T cell response against TAMs that highly express this asparaginyl endopeptidase. This was demonstrated by a ⁵¹Cr release assay, in which splenocytes isolated from mice successfully immunized with this vaccine were effective in killing RAW macrophages, which expressed high levels of legumain after culture with cytokines IL-4, IL-10, and IL-13; however, these

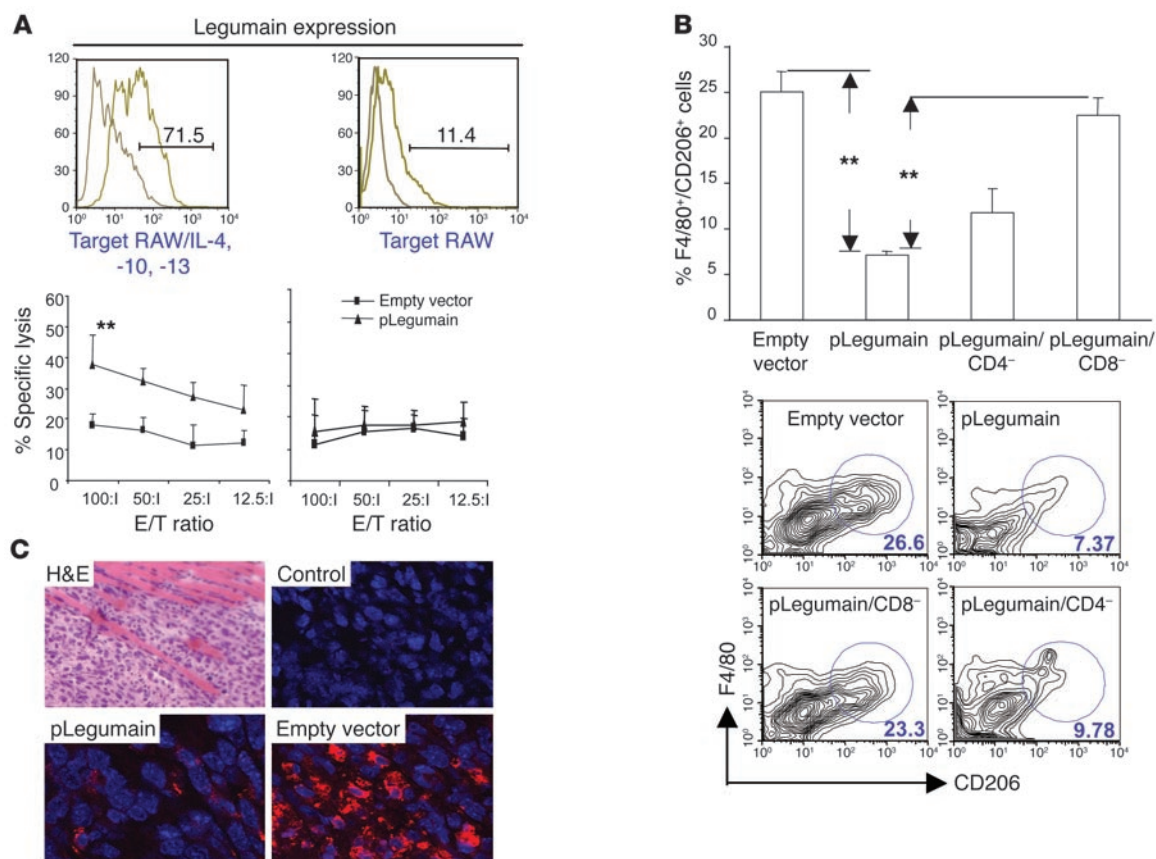
Figure 2

Targeting of legumain-expressing cells results in suppression of tumor progression. **(A)** Schematic of DNA vaccines constructed with the pCMV/myc/cyto vector backbone where the legumain gene was fused to the C terminal of mutant polyubiquitin. The entire fragment was inserted, and protein expression was demonstrated by Western blotting. Mu-legumain, murine legumain. **(B)** Prophylactic model: The vaccination schedule was designed for 3 immunizations at 1-week intervals followed by an i.v. challenge with 2×10^5 D121 non-small cell lung cancer cells and 5×10^4 CT26 colon cancer cells and mammary fat pad injection with 7×10^3 4T1 breast cancer cells. Lung weights were determined 24 days (D121 or CT26) or 30 days (4T1) after tumor cell challenge and analyzed in each group. Differences between the 2 control groups (PBS and/or empty vector) and the treatment group were statistically significant; ** $P < 0.005$. Pre-challenge lung weight, 0.2 g. **(C)** Therapeutic model: Groups of BALB/c mice ($n = 8$) were initially injected in the mammary fat pad with 7×10^3 4T1 breast cancer cells and thereafter vaccinated 3 times on days 3, 7, and 11 with PBS, empty vector, or the pLegumain vaccine, respectively, and primary tumors excised on day 12. Survival plots represent results for 8 mice in each of the treatment and control groups. The difference between the empty vector control group and the treatment group was statistically significant; ** $P < 0.005$.

same splenocytes failed to induce cytotoxic killing of cells that lacked legumain expression (Figure 3A), indicating the specificity of this T cell response against legumain. Additionally, the same result was obtained using legumain-transfected cells as target cells in ⁵¹Cr release assays (Supplemental Figure 1; available online with this article; doi:10.1172/JCI27648DS1). Furthermore, the results depicted in Figure 3B demonstrate a dramatic decrease in the F4/80⁺/CD206⁺ macrophage population after legumain-based DNA vaccine treatment. These data were also confirmed by immunohistochemical staining, as shown in Figure 3C.

MHC class I-restricted CD8⁺ CTLs are specifically active against TAMs. In gaining some insight into the immune mechanisms involved in the cytotoxic activity against TAMs, we found that DCs in Peyer's patches of successfully immunized mice were activated 3 days after vaccination with pLegumain, as indicated by the upregulated DC activation markers CD40, CD80, and MHC class I (Figure 4A). Furthermore, CD8⁺ T cell activation was found to be specific for legumain, as indicated by double staining for IFN- γ and CD8 on splenocytes obtained from successfully vaccinated mice (Figure 4B) and by the specific release of IFN- γ by activated T cells stimulated with legumain-positive cells (Figure 4C). In addition, in vivo immune depletion of CD4⁺ or CD8⁺ T cells revealed that only CD8⁺ T cells play a major role in the specific cytotoxic killing of TAMs, since only their depletion completely abrogated this killing effect. This specific cytotoxicity was MHC class I antigen restricted, as killing was specifically inhibited by anti-H-2D^d/H-2K^d antibodies (Figure 4D). Taken together, our results suggest that the legumain-based DNA vaccine first activated DCs in Peyer's patches, after which these cells presented legumain peptides through the MHC class I antigen pathway to the TCR on activated CD8⁺ T cells, resulting in a specific cytotoxic CD8⁺ T cell response abrogating TAMs.

Abrogation of TAMs in the tumor stroma reduces the release of tumor growth factors and proangiogenic factors as well as inhibiting tumor cell migration and metastases. TAMs can influence tumor metastasis in several ways, as they secrete a wide variety of tumor growth factors, proangiogenic factors, and tumor-associated enzymes that

**Figure 3**

TAM population in the tumor stroma is decreased by CD8⁺-specific CTLs induced by the legumain-based DNA vaccine. **(A)** RAW macrophage cells, which highly express legumain after culturing with 10 ng/ml IL-4, IL-10, and IL-13, served as target cells in a 4-hour ⁵¹Cr release assay. Splenocytes isolated from mice immunized with the pLegumain vaccine were shown to effectively kill RAW cells treated with these cytokines in vitro at different effector-to-target (E/T) cell ratios but failed to induce cytotoxic killing of unstimulated RAW cells lacking legumain expression. **(B)** The percentage of TAM populations with specific macrophage markers (CD206 and F4/80) in tumor tissue with or without vaccination was detected by flow cytometry. The percentage of TAM populations among tumor tissue cells isolated from mice treated with our DNA vaccine was shown to be reduced; however, there was no decrease in TAM populations isolated from mice treated with either empty vector or pLegumain following CD8⁺ T cell depletion (****P** < 0.005). **(C)** The results of flow cytometry were confirmed by immunohistochemical staining evaluated by confocal microscopy. The population of TAMs in the tumor stroma was dramatically decreased after vaccination. 4T1 cancer cells are shown in blue and TAMs in red. Magnification, ×50 (H&E) and ×350 (control, empty vector, and pLegumain).

stimulate tumor angiogenesis and tumor growth and metastasis. In an effort to assess whether the elimination of TAMs actually reduced the release of some of these factors, serum and tumor tissue cells were collected from vaccinated mice and from suitable control animals. Freshly isolated tumor cells were cultured and their supernatants collected at 24 and 48 hours, respectively. An ELISA, performed to quantify TNF- α , VEGF, and TGF- β , indicated a significant reduction in TNF- α and VEGF in both tumor cell supernatants and mouse serum; however, TGF- β was significantly reduced only in serum but not in cell supernatants (Figure 5A). Immunohistological staining confirmed a decrease in the expression of these factors in tumor tissue (Figure 5B). In addition, a significantly decreased tumor cell migration was found when treatment and control groups were compared (Figure 5C) in a migration and invasion assay, which indicated that these characteristics of tumor cells changed after the vaccine-induced remodeling of the tumor microenvironment caused by the reduction in TAMs. The ability to form tumor metas-

tases was confirmed in an in vivo experiment as the metastasis scores and lung weights — measured 24 days after primary tumor excision in a therapeutic setting, as described in Methods — decreased significantly when compared with the 2 control groups (Figure 5D).

Elimination of TAMs in the tumor stroma results in reduction of tumor angiogenesis. Importantly, there also was a marked antiangiogenic effect after elimination of TAMs in the tumor stroma, particularly since these M2 macrophages produced a wide range of pro-angiogenic factors. This was established by Matrigel assays that detected new blood vessel growth in vivo, an effect that could be quantified by staining the endothelium with FITC-labeled isolectin B4. These results clearly show that vessel growth was significantly reduced after vaccination with pLegumain (Figure 6B). It was also clearly indicated that much more blood vessels grew in Matrigel plugs in mice immunized with empty vector after evaluation by digital imaging and with Masson's trichrome staining (Figure 6A). Furthermore, an immunochemical histology assay

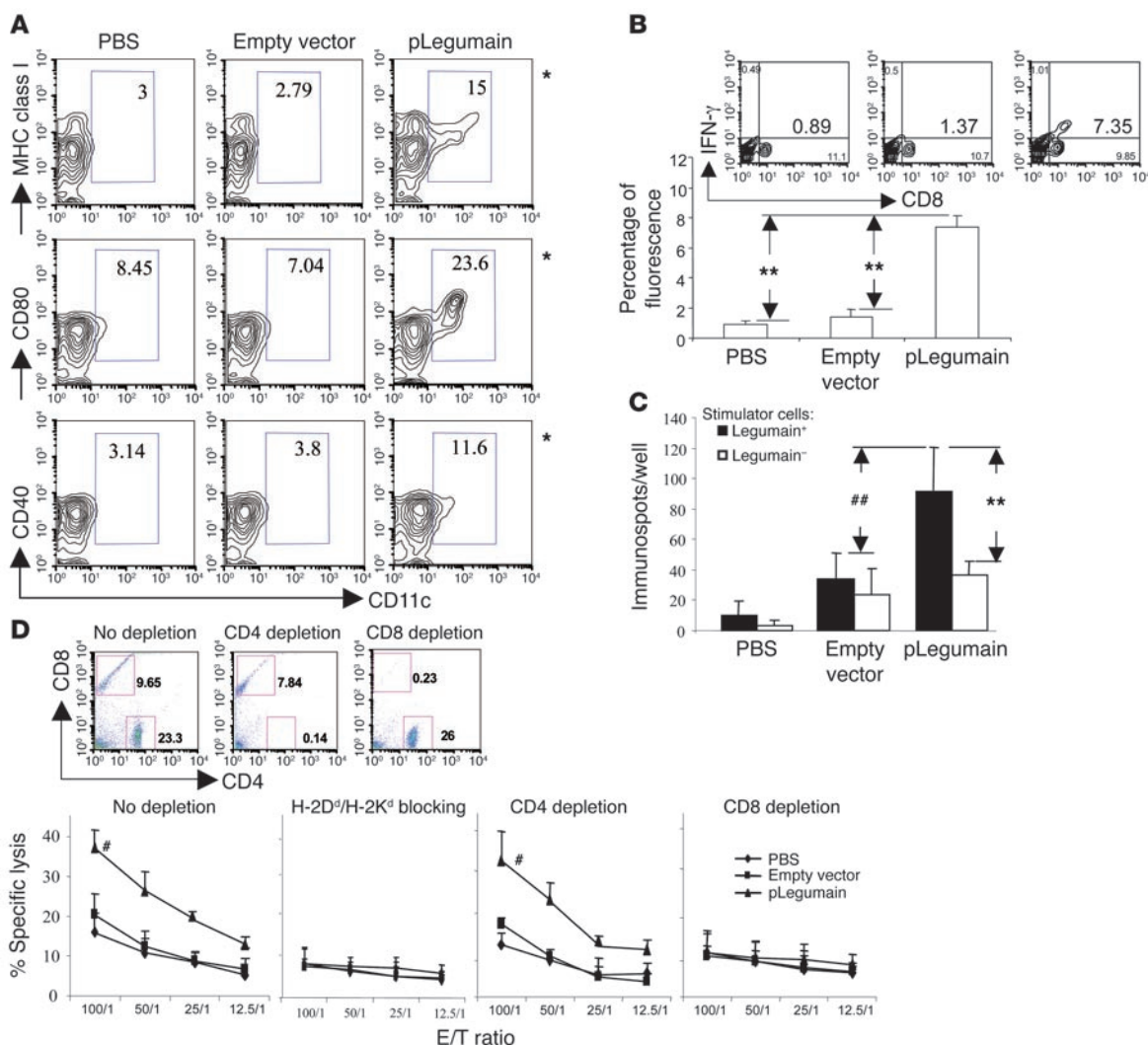


Figure 4

MHC class I antigen-restricted specific CD8⁺ T cell response against legumain-expressing cells. **(A)** DNA vaccination enhances expression of costimulatory molecules by DCs. Lymphocytes from Peyer's patches obtained 3 days after vaccination were stained with FITC-labeled anti-CD11cAb in combination with PE-conjugated anti-CD80, anti-MHC class I, or anti-CD40 Abs. **P* < 0.05 compared with control groups. **(B)** Intracytoplasmic IFN-γ release of CD8⁺ T cells was measured by FACS analysis. ***P* < 0.005 compared with control groups. **(C)** Production of specific IFN-γ was verified at the single-cell level by ELISPOT. This is indicated for lymphocytes from immunized mice restimulated with either legumain⁺ 4T1 tumor tissue cells or legumain⁻ 4T1 cells, as indicated by the number of immunospots formed per well. ***P* < 0.005 compared with treatment group without stimulation; ##*P* < 0.005 compared with control groups. **(D)** Splenocytes isolated from treated mice were effective in killing TAMs according to a ⁵¹Cr release assay (#*P* < 0.01 compared with control groups). Inhibition experiments with Abs against H-2K^d/H-2D^d MHC class I antigens showed that T cell-mediated tumor cell lysis was MHC class I antigen restricted. Furthermore, in vivo depletions of CD4⁺ or CD8⁺ T cells indicated that lymphocytes isolated from vaccinated mice, which were thereafter depleted of CD8⁺ T cells, failed to induce cytotoxic killing of target cells. However, depletion of CD4⁺ T cells did not abrogate cytotoxic killing of these same target cells. **P* < 0.01 compared with PBS or empty vector group.

was performed to assess the type of cells that actually migrated into the Matrigel plugs. The confocal images taken indicated that endothelial cells expressing CD31⁺ or macrophages expressing CD68⁺ grew or migrated into Matrigel plugs to a considerably greater extent in the empty vector control group than in the vaccine treatment group (Figure 6C).

Discussion

This study establishes the new paradigm whereby a reduction in the density of TAMs in the tumor stroma decreases the release of factors potentiating tumor growth and angiogenesis. This, in

turn, remodels the tumor microenvironment so as to markedly suppress tumor cell proliferation, vascularization, and metastasis. However, targeting TAMs in the tumor stroma raises the concern that their abrogation could interfere with the normal immunological functions of these important components of the innate immune system. We addressed this question in view of the fact that circulating monocytes are versatile precursors with the ability to differentiate into the various forms of specialized macrophages (3). In fact, the cytokine milieu profoundly affects the differentiation and function of tissue macrophages, and their functional polarization has been defined (3, 20, 21). Thus, macrophages

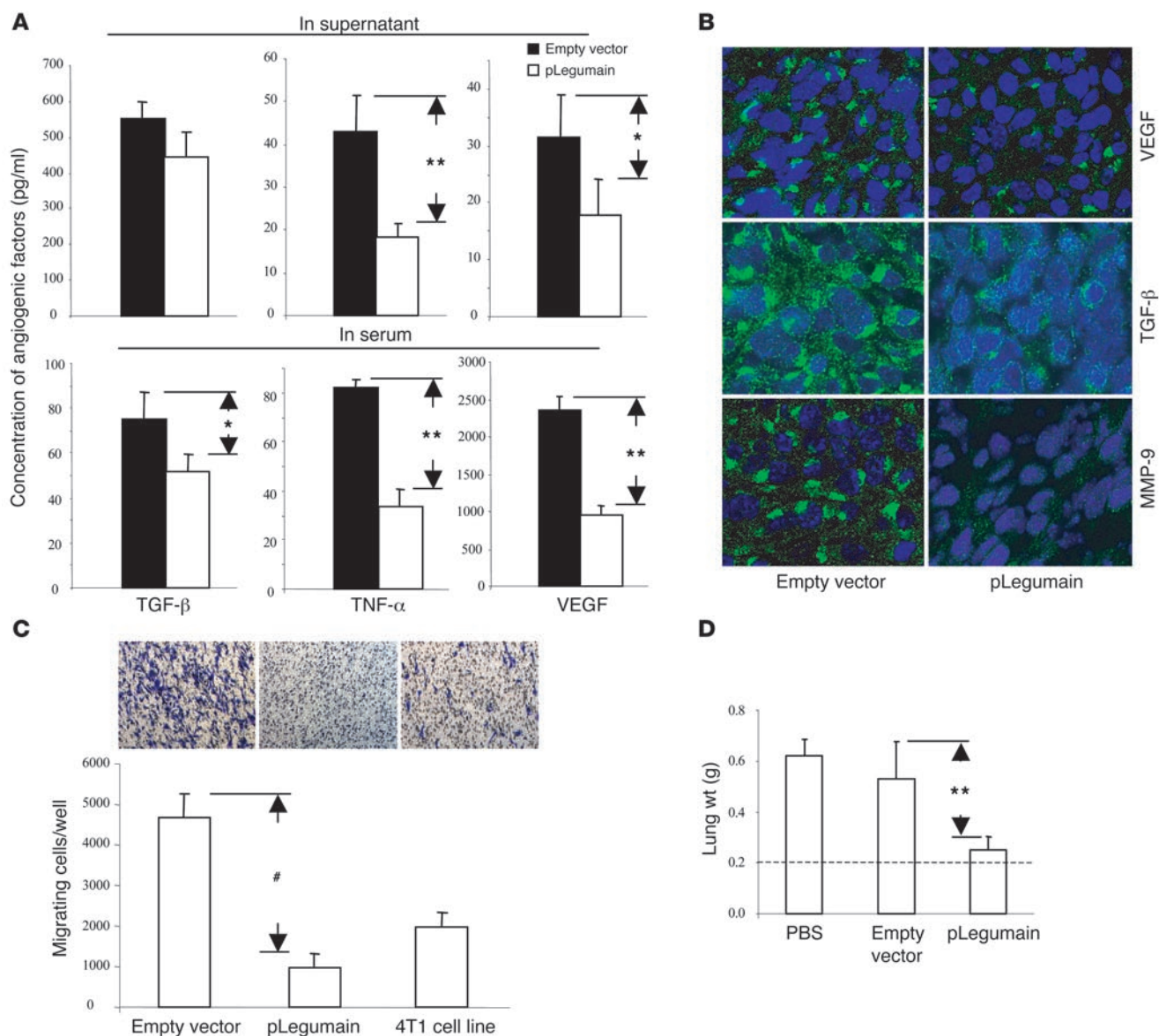


Figure 5

Abrogation of TAMs results in decreases of growth factor release, tumor cell migration, and metastases. **(A)** The vaccine decreased the release of growth factors by TAMs. 4T1 breast tumor tissue and mouse serum were harvested 12 days after vaccinations and tumor cell challenge. After 24 or 48 hours culturing, the supernatants of tumor tissue cells were harvested, and the concentrations of TGF- β , TNF- α , and VEGF in serum or supernatants measured by ELISA. There were significant differences between the treatment and control groups. $*P < 0.01$; $**P < 0.005$. **(B)** Immunohistochemical staining was performed to determine expression of these growth factors in the tumor microenvironment. In the vaccine treatment groups, VEGF, TGF- β , and MMP-9 release was decreased after a reduction in TAMs, compared with the empty vector groups. The growth factors are shown in green and 4T1 breast cancer cells in blue. **(C)** A Transwell migration assay was performed to determine tumor cell migration after vaccination. The number of migrating cells isolated from 4T1 tumor tissue was markedly reduced after vaccination. $\#P < 0.001$ compared with the empty vector group. **(D)** In vivo experiments were performed to determine the ability of mice to form 4T1 tumor metastases. The mice were treated with the vaccine within the therapeutic setting as described above. Tumor metastasis scores and lung weights were measured 25 days after primary tumor excision. The metastasis scores are expressed as the percentage of lung surface covered by fused metastatic foci; 0: none; 1: $<5\%$; 2: $5\text{--}50\%$; 3: $>50\%$. Scores for $n = 8$ mice/group were: PBS, 3, 3, 3, 3, 3, 3, 2, 2; empty vector, 3, 3, 3, 3, 3, 3, 3, 2; pLegumain, 2, 2, 1, 0, 0, 0, 0, 0. Differences in lung weights between the group of mice treated with vaccine and all control groups were statistically significant ($**P < 0.005$). Magnification, $\times 350$ (B), $\times 50$ (C).

activated by bacterial products and Th1 cytokines are regarded as being of the M1 phenotype, i.e., classically activated macrophages with high bactericidal activity and cytotoxic function against tumor cells. However, macrophages activated by such Th2

cytokines as IL-4 and IL-13 or immunosuppressors such as vitamin D3 and IL-10 are classified as macrophages with an M2 phenotype: low cytotoxic functions but high tissue-remodeling activity. Whereas M1 cells have immunostimulatory properties and

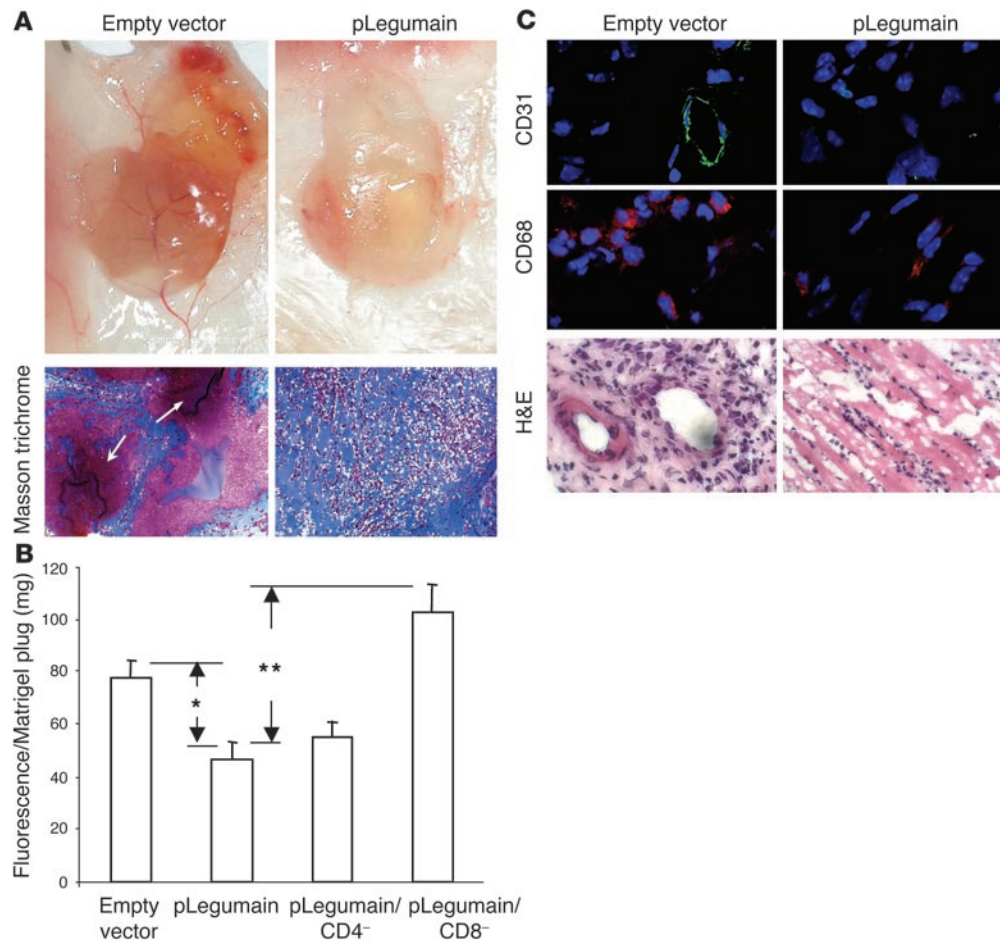


Figure 6
Elimination of TAMs results in a reduction in tumor angiogenesis. Suppression of VEGF-induced angiogenesis: BALB/c mice were vaccinated with empty vector, pLegumain, or pLegumain after either CD8⁺ or CD4⁺ T cell depletion in vivo. One week after the last vaccination, Matrigel was implanted s.c. into the midline of the abdomen of mice. Vascularization was induced by VEGF or bFGF. **(A)** The images were taken by a digital camera 6 days after Matrigel plug implantation. Additionally, the section of Matrigel plugs stained with Masson's trichrome indicate blood vessel growth in Matrigel plugs, as indicated by arrows. Magnification, $\times 50$. **(B)** Quantification of vessel growth was performed after in vivo staining of endothelium with FITC-labeled isolectin B4 and evaluation by fluorimetry. There was a decrease in the VEGF-induced neovasculture only after vaccination with the vector encoding legumain but not after vaccination with the empty vector or with pLegumain after depletion of CD8⁺ T cells. $^{**}P < 0.005$, $^{*}P < 0.01$ compared with the legumain treatment group. **(C)** Immunohistochemical staining was performed and evaluated by confocal microscopy. The cross-sections of Matrigel plugs were stained to determine the cell type that grew in or migrated into these plugs. The images indicate that endothelial cells with the CD31 marker or macrophages with the CD68 marker grew in or migrated into Matrigel plugs as indicated (magnification, $\times 350$). H&E staining served as a control (magnification $\times 50$).

defend the host against pathogenic infections, M2 cells attenuate acute inflammatory reactions, potently scavenge cellular debris, and secrete a variety of pro-growth and angiogenic factors essential for the repair of injured tissues. In addition, macrophages derived from healthy or inflamed tissue are capable of lysing tumor cells, expressing immunostimulatory cytokines, and presenting tumor-associated antigens to stimulate the proliferation and antitumor functions of T and NK cells. However, M2 macrophages, such as TAMs, show reduced levels of these activities. This may be the result of their exposure to tumor-derived antiinflammatory molecules such as IL-4, IL-10, TGF- β 1, and prostaglandin E₂ (22, 23). Indeed, this finding prompted Mantovani and col-

leagues to suggest that exposure to IL-4 and IL-10 may induce monocytes in tumors to develop into polarized type II or M2 macrophages (24). To the extent that they have been investigated thus far, differentiated mature TAMs have a phenotype and function similar to those of type II macrophages (6). Therefore, cytokines present in the tumor microenvironment have the potential to promote and orient the differentiation of recruited mononuclear phagocytes (25). Indeed, a growing body of evidence indicates that TAMs are skewed toward M2 macrophages in the tumor microenvironment and produce a variety of pro-tumor growth and angiogenic factors as well as immunosuppressive molecules (1, 6, 26, 27). Thus, the presence of TAMs at the tumor site and the continuous expression and release of their products may favor, rather than antagonize, tumor progression and metastasis.

In our study we demonstrated that TAMs express abundant levels of CD206, a mannose receptor that is upregulated on M2 macrophages following exposure to IL-4 and IL-13 (27–30). We also established simultaneously that this population of macrophages expressed high levels of legumain. Importantly, we found that Th2 cytokines IL-4, IL-10, and IL-13 could upregulate the expression of CD206 and

legumain on the macrophage cell line RAW. This finding can best be understood when one considers that macrophages are derived from peripheral blood and differentiate into M2 macrophages once they are recruited into tumor sites where IL-4, IL-13, and IL-10 are released by tumor cells and tumor stromal cells (3, 19, 29, 31). Thus, targeting of M2 macrophages expressing legumain not only benefits suppression of tumor growth and metastases but also maintains the normal functions of macrophages with the M1 phenotype.

The relationship between infiltration by TAMs and prognosis in tumor patients has also been indicated by several studies (1, 5, 32, 33), which concluded that the greater the macrophage infiltration, the worse the prognosis. Several lines of evidence indicate that a



symbiotic relationship exists in the tumor stroma between cancer cells and TAMs, whereby cancer cells attract TAMs and sustain their survival, while TAMs respond to tumor-derived molecules by producing important growth factors and extracellular matrix enzymes, which, in turn, stimulate tumor proliferation, angiogenesis, and invasion of surrounding tissues (18, 22, 26, 34). Thus, the attenuation of TAMs in the tumor environment can serve as an effective strategy to remodel the tumor stroma and to alter the tumor microenvironment (35).

In our study, a DNA construct encoding legumain evoked a robust CTL response against this asparaginyl endopeptidase, which functions as a stress protein that is highly overexpressed by TAMs. This immune response was shown to be MHC class I antigen restricted and CD8⁺ T cell specific. Importantly, our data also demonstrated that after immunization with the legumain-based DNA vaccines, the density of F4/80⁺/CD206⁺ macrophages, i.e., TAMs, decreased dramatically. Furthermore, a variety of factors such as VEGF, MMP-9, and TGF- β that are released by TAMs were shown to be at low levels in both the supernatant of cultured tumor cells and mouse serum. Thus, it is well known that VEGF and metalloproteinase MMP-9 play important roles during the formation of the tumor vasculature and initiation of tumor angiogenesis. TAMs are important in this regard, since they produce both VEGF and MMP-9 (36). Progressively intensifying angiogenesis is associated with the upregulated expression of VEGF (37) and extracellular proteases, such as MMP-9 (8, 36), whereas TGF- β is known as an important growth factor involved in the migration of tumor cells toward blood vessels. In fact, TGF- β can provide proliferative and antiapoptotic signals to tumor cells as well as activate urokinase-type plasminogen activators (uPAs), which might contribute to the extracellular matrix breakdown that is required for vascular invasion to occur (38). Significantly, our data demonstrate that once TAMs were abrogated in the tumor tissue by specific CD8⁺ CTLs, the tumor cells changed their character by becoming less malignant and less invasive. Also, the formation of a neovasculature in tumor tissues was reduced, since all of the factors released by TAMs that contribute to tumor angiogenesis were drastically reduced. Additionally, TAMs were reported to be involved in immune suppression and tolerance in the tumor microenvironment (39). It is also well known that TAMs may inhibit T cell responses by inducing apoptosis of activated T cells via upregulation of NO, PGs, TNF- α release, and arginase activity (40, 41). These may be some of the reasons for tumor immune tolerance, a notion also supported by our data. After abrogation of TAMs, the specific CD8 T cell activity was markedly upregulated, further supporting the contention that our anti-TAM approach could be a good strategy to break immune tolerance against tumors.

Furthermore, our hypothesis that a therapeutic approach using a legumain-based DNA vaccine to target TAMs holds much promise was strongly supported by data obtained in 3 tumor metastasis models used in our study. Thus, in the 4T1 spontaneous mouse breast carcinoma metastasis model, a significant increase in life span was obtained, as 75% (6/8) mice survived up to 3 months after 4T1 tumor cell inoculation into the mammary gland, once surgical resection of the primary tumor was followed by treatment with the legumain-based DNA vaccine. It was even more impressive that 62% (5/8) mice revealed no lung metastases at all. Similar results were obtained in prophylactic settings in the other 2 tumor models, i.e., D121 non-small cell lung carcinoma and CT26 colon

carcinoma. These additional confirmatory data strengthen our contention that targeting of TAMs to remodel the tumor microenvironment might be a universal antitumor strategy for suppressing tumor cell invasion and metastases by reducing the concentration of factors released by TAMs that otherwise promote tumor growth and metastasis.

In summary, we critically evaluated the antitumor efficacy of targeting TAMs via the induction of a specific CD8⁺ T cell response against legumain, which we identified for the first time as being a highly overexpressed target molecule on TAMs. In these experiments, we demonstrated that abrogation of TAMs in tumor tissues effectively decreased the release of several pro-tumor growth and angiogenic factors. It is likely that such an antitumor strategy could be widely applicable and relevant for possible clinical applications.

Methods

Animals, bacterial strains, and cell lines. Female BALB/c and C57BL/6 mice, 6–8 weeks of age, were purchased from The Scripps Research Institute Rodent Breeding Facility. The double-attenuated *S. typhimurium* strain RE88 (*aroA*[−]*;**dam*[−]) was obtained from Remedyne Corp. The murine CT26 colon cancer cell line was kindly provided by I.J. Fidler (MD Anderson Cancer Center, Houston, Texas, USA), and the murine D121 non-small cell lung carcinoma cells were a gift from L. Eisenbach (Weizmann Institute of Science, Rehovot, Israel). The murine 4T1 breast carcinoma cells were kindly provided by Suzanne Ostrand-Rosenberg (University of Maryland, College Park, Maryland, USA).

Immunohistochemical analyses. These were performed on 4T1 tumor tissues and Matrigel plug sections. Legumain expression of macrophages was identified on 4T1 tumor tissue sections with biotinylated rat anti-mouse CD68 mAb (BD Biosciences – Pharmingen), with GFP-conjugated streptavidin being the secondary reporter reagent. Rabbit anti-legumain antiserum was prepared by immunization with purified human legumain produced in *Escherichia coli*. (10) The reaction was visualized with Texas red-conjugated streptavidin. Additionally, 4T1 tumor tissue sections and Matrigel plug sections were fixed and stained with MMP-9, VEGF, TGF- β , and F4/80 antibodies (eBioscience and Santa Cruz Biotechnology Inc.) in 4T1 tumor tissue section, while CD68 and CD31 Abs (BD Biosciences – Pharmingen) were used in Matrigel plug sections. All tissue sections were visualized with Texas red- or GFP-conjugated streptavidin as the secondary reporting reagent, and the slides were analyzed with laser scanning by confocal microscopy (Bio-Rad). All the images were captured by a SPOT Cooled Color Digital Camera System (Diagnostic Instruments Inc.).

Vector construction, protein expression, and transformation of *S. typhimurium* with DNA vaccine plasmids. Two constructs were made based on the vector pCMV (Invitrogen). The pLegumain construct consisted of polyubiquitinated, full-length murine legumain. The empty vector construct served as a control. Protein expression of legumain was demonstrated by Western blotting with a polyclonal rabbit anti-murine legumain Ab as well as anti-murine β -actin Ab (Santa Cruz Biotechnology Inc.) as a loading control. The specific protein was detected with a goat anti-rabbit HRP-conjugated IgG Ab (Bio-Rad). Attenuated *S. typhimurium* (*aroA*[−]*;**dam*[−]) were transduced with DNA vaccine plasmids by electroporation as described in our previous publications (42, 43).

Immunization and tumor cell challenge. For the prophylactic model, BALB/c or C57BL/6 mice were each divided into 3 experimental groups ($n = 8$) and immunized with PBS, empty vector, or pLegumain. All mice were challenged by i.v. injection with 5×10^4 CT26 cells (BALB/c) or 2×10^5 D121 cells (C57BL/6) or injected in the mammary gland fat pad with 7×10^3 4T1 cells (BALB/c), 1 week after the last immunization, to induce either experimental or spontaneous pulmonary metastases. The lung weights



in experimental or control groups were determined 24 days after tumor cell challenge. For the therapeutic model, BALB/c mice were divided into 3 experimental groups ($n = 8$) and first injected in the fat pad with 7×10^3 4T1 cells on day 0 and then immunized 3 times with DNA vaccine starting on days 3, 7, and 11, and primary tumor was excised on day 12. The experiment was terminated 24 days after primary tumor excision to determine lung weights and metastasis scores or mouse survival rates.

In vivo depletion of CD4⁺ or CD8⁺ T cells, cytotoxicity, and ELISPOT assays. Analysis of the depletion of CD4⁺ or CD8⁺ T cells in vivo was performed as previously described (44). Cytotoxicity was measured and calculated by a standard ⁵¹Cr release assay as previously reported (45). ELISPOT assays were performed with an ELISPOT kit (BD Biosciences — Pharmingen) according to instructions provided by the manufacturer.

In vivo Matrigel angiogenesis assay. Matrigel was used for evaluating the suppression of angiogenesis after vaccination. Briefly, BALB/c mice were injected s.c. 2 weeks after the last vaccination, in the sternal region, with Growth Factor Reduced BD Matrigel (BD Biosciences) containing bFGF-2 (200 ng/plug) and 4T1 tumor cells (5×10^3 /plug) that were previously irradiated with 1,000 Gy. The endothelium was stained 6 days after Matrigel implantation by i.v. injection with *Bandeiraea simplicifolia* lectin I (isolectin B4), conjugated with fluorescein (Vector Laboratories). This was done along with staining the endothelium of control animals, and 30 minutes later, mice were sacrificed, Matrigel plugs extracted, and fluorescence evaluated by fluorimetry. Additionally, the Matrigel plugs were removed 6 days after Matrigel implantation, fixed in Bouin's solution for 24 hours, and then embedded in paraffin. All tissues were sectioned, mounted onto slides, and stained with Masson's trichrome. All of the images were captured by a SPOT cooled color digital camera system (Diagnostic Instruments Inc.)

Flow cytometry. DC cell markers were determined by staining freshly isolated lymphocytes from successfully vaccinated mice and control mice with PE-labeled anti-CD11c Ab in combination with FITC-conjugated anti-CD40, anti-CD80 Ab, and Abs against MHC class II antigen. Macrophages bearing high levels of CD206⁺ and F4/80⁺ were quantified by 2-color flow analysis. Tumor cells were isolated from successfully vaccinated BALB/c mice and then stained with anti-CD206 Ab conjugated with PE (Cell Sciences),

anti-F4/80 Ab conjugated with APC, and anti-legumain Ab conjugated with FITC, followed by FACS analyses. All antibodies were purchased from BD Biosciences — Pharmingen. IFN- γ release at the intracellular level was determined in lymphocytes of Peyer's patches obtained 3 days after one-time immunization and stained with APC-conjugated anti-CD8 Ab. Cell were fixed, permeabilized, and subsequently stained with PE-labeled anti-IFN- γ Ab to detect intracellular expression of IFN- γ .

Migration assay. Cell migration assays were performed using modified Boyden chambers (Transwell; Corning Inc.). Transwell migration assays were performed with tumor cells harvested from tumor tissue of either vaccine-treated or control groups of mice. After 4 hours culture, the cells on the lower surface of wells were fixed with 1% paraformaldehyde, stained with 1% crystal violet, and counted (46).

Statistics. The statistical significance of differential findings between experimental groups and controls was determined by Student's *t* test. Findings were regarded as significant if 2-tailed *P* values were less than 0.05. Kaplan-Meier analysis was used to evaluate the survival of mice.

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